CELL SPECIFIC REGULATION OF SONIC HEDGEHOG IN ADULT STOMACH: UNDERSTANDING MECHANISMS LEADING TO GASTRIC ATROPHY/METAPLASIA

by

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They began my education,
They motivated me to continue it,
They will always contribute to it.

To Amma, Nana, Mom, Dad, Varsha, and Bharath,
the most loving and encouraging family in the world

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As the day is coming to a close, I am about to finish my acknowledgements and with them this dissertation. My years at University of Michigan were full of excitement and wonder, and I believe that completion of this thesis is one of the least significant things that have happened to me. The degree alone does not come close to the importance of meeting the people that I was lucky t meet and learning the things that I have while I have been here. And I daresay that I am closer to being a scientist now than I was four and a half years ago.

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INTRODUCTION

Having a stomach is the main reason we do not need to eat constantly. Our stomach is highly elastic and can stretch to accommodate enough food to satisfy our body's needs for several hours. Storage is not a primary function of the human stomach. The stomach aids in digestion and absorption by producing acid, digestive enzymes, mucins, and intrinsic factor. Acid produced by the stomach kills bacteria in the food, under these low pH conditions inactive pepsinogen is converted to active pepsin. About 10-20% of total protein digestion occurs in the stomach. Pepsin acts on proteins breaking them into polypeptides. To protect itself from being digested by actions of acid and pepsin, the stomach coats itself with mucous. Muscular movements of the stomach along with acid, mucous and pepsin breaks down, mixes and liquefies the food to chyme, which passes through the opening of the pyloric sphincter into the small intestine for further digestion and absorption.

The work in this thesis concerns gastric physiology and pathophysiology. First, I will introduce the morphological organization of cells in the corpus, and subsequently their biochemical and physiological properties. I will then, introduce the hedgehog signaling pathway and its role in patterning the

embryonic and adult stomach and discuss recent data regarding the regulation of Sonic Hedgehog (Shh) in the adult stomach.

Morphology of the stomach

Around mid fourth week of human embryonic development, the caudal part of the foregut slightly dilates appearing as a fusiform structure oriented in the median plane. The fusiform structure soon enlarges ventrodorsally and the dorsal border grows faster than the ventral border and this demarcates the greater curvature of stomach. As the stomach acquires its adult shape it rotates 90^{o} clockwise along the longitudinal axis. Hence the ventral border lies on the right and the dorsal border or the greater curvature moves to the left of the abdomen.

The human stomach consists of four regions, the cardia, fundus, corpus (body) and antrum (Fig. 1.1 A). The cardia is the region located near the gastro-esophageal junction. The mouse stomach differs from the human stomach in this region. The mouse stomach consists of a forestomach region with squamous epithelium connecting the fundus to the esophagus (Fig. 1.1 B). The fundus and corpus (body) contain the acid-secreting parietal cells and zymogenic cells whereas; the antrum contains mostly mucous cells.

Four distinct layers can be seen in tissue sections throughout the stomach of mice and humans: the mucosa (epithelium, lamina propria, and muscularis mucosa), submucosa, muscularis externa (inner most obliquely-oriented muscle layer, circular muscle layer and outer longitudinal muscle) and serosa. The

surface mucosa consists of a single layer of columnar epithelium invaginating into lamina propria producing blind tubular units called glands (Fig 1.2) (Karam and Leblond 1992). Each unit or gland in the corpus consists of four regions pit, isthmus, neck and base. On average, each gland consists of about 200 cells distributed along the four regions (Karam and Leblond 1992). Based on detailed light and electron microscopy (EM) and ^{3}H-thymidine labeling analysis, 11 different cell types have been described in a gland (Fig. 1.3) (Karam and Leblond 1992; Karam and Leblond 1993). The pluripotent stem cell resides in the isthmus region and is identified under EM as a granule free cell containing a nucleus with diffuse chromatin, large reticulated nucleoli, scanty cytoplasm and numerous free ribosomes (Karam and Leblond 1993). The pluripotent stem cell is believed to give rise to three different types of progenitor cells (pre-pit, pre-neck, and pre-parietal cells), as well as entero-endocrine cells, and caveolated cells (Fig. 1.3). These cells originating from the isthmus then migrate in an outward and /or inward direction giving rise to different cell lineages lining the gland (Fig. 1.2, 1.3) (Karam and Leblond 1993).

Surface pit cells

The pre-pit progenitor cells in the isthmus mature to give rise to pre-pit cells which contain few mucous secretory granules scattered in the cytoplasm. The pre-pit cell migrates outwards along the pit wall, accumulates secretory granules in the apical ectoplasm differentiating into the surface pit cell (Fig. 1.4). Once the surface pit cells reach the gastric luminal surface, they are extruded

into the lumen or are phagocytosed by neighboring cells. The average turnover time for pit cells is 3 days (Karam and Leblond 1993).

Mucous neck and Zymogenic cells

The pre-neck cells derived from granule free cells in the isthmus region migrate towards the base of the gland to give rise to the neck cells. The mucous neck cells are derived from pre-neck cell precursors, and are characterized by the presence of dense mucous granules. They spend 7-14 days in the neck region and then migrate towards the blind end of the gastric unit (Fig. 1.4). As they move closer to the base, these cells transform into pre-zymogenic cells. The pre-zymogenic cells further migrate towards the base of the gland producing larger pepsinogen-containing granules, as they differentiate into zymogenic cells (Fig. 1.4). At the base, zymogenic cells undergo necrosis releasing their contents into the lumen or undergo apoptosis and are phagocytosed by neighboring zymogenic cells or tissue macrophages. The turn over time for zymogenic cells is 194 days (Karam and Leblond 1993).

Entero-endocrine and Caveolated cells

The entero-endocrine cells directly differentiate from the granule free stem cells and migrate in both directions towards lumen and base of the gland. These cells retain little ability to divide and turn over every 2 to 3 months (Fig. 1.3) (Karam and Leblond 1993).

The caveolated cells, also known as the multi-vesicular, brush, or fibrillo-vesicular cells, are characterized by the presence of prominent microvilli and long

caveoli. These cells arise from the isthmus region and migrate both in outward and inward direction, but rarely proceed as far as the base (Fig. 1.3) (Karam and Leblond 1993).

Parietal cells

Pre-parietal cells in the isthmus region are recognized by apical microvilli and canaliculi. There are three variants of pre-parietal cells: variant (I) a pre-parietal precursor with no granules, variant (II) a pre-parietal precursor with few secretory granules (similar to the pre-pit cell granules, these develop from pre-pit cell progenitors);and variant (III) a pre-parietal cell precursor with granules similar to that of pre-neck cells, which develop from pre-neck progenitors (Fig 1.4) (Karam 1993).

Development of pre-parietal cells into parietal cells occurs by increasing the surface area of the apical plasma membrane, accumulating tubular vesicles in the cytoplasm, forming a canaliculus, and increasing both the number of mitochondria and cell size. Once the parietal cells are produced in the isthmus, they migrate outwards towards the surface pit and inwards towards the neck and base of the unit. The secretory activity of the parietal cells varies along the gland axis. The young cells near the isthmus and neck region are more active than the old parietal cells near the pit and base (Karam, Yao et al. 1997). At the surface or at the base, the parietal cells either are extruded into the lumen or are phagocytosed by neighboring surface pit or zymogen cell respectively. The turnover time of parietal cells is 54 days (Karam 1993).

The antral glands are mainly made up of mucous cells; the cells at the base of the gland consist of G-cells which secrete gastrin and the D-cells that secrete somatostatin (Karam, Tomasetto et al. 2008). Gastrin and somatostatin are the two major peptides involved in the regulation of gastric acid secretion.

Physiology of the stomach

The ability to secrete acid in a well-orchestrated manner requires the interplay of functions of the entero-endocrine cells and the parietal cell, and involves a complex network of endocrine, paracrine, and neural factors. The major stimulants of gastric acid secretion include gastrin, histamine and acetylcholine (ACh), which activate the parietal cell to secrete acid into the lumen of the stomach (Fig. 1.5). Gastrin, histamine and ACh bind to well-defined receptors on the basolateral membrane of parietal cells. The Cholecystokinin-B (CCK_B) receptor binds gastrin, the histamine-2 (H_2) receptor binds histamine, and the muscarinic-3 (M3) receptor binds ACh. Gastrin released from G-cells can stimulate acid secretion directly from parietal cells or can stimulate the release of histamine from ECL cells to stimulate acid production (Hersey and Sachs 1995). The Neuro-transmitter ACh stimulates acid secretion directly from parietal cells while, the pituitary adenylate cyclase activating polypeptide (PACAP) induces production of histamine by binding to PACAP-1 receptors on ECL cells to stimulate acid secretion (Ozawa, Aono et al. 1997; Zeng, Kang et al. 1998; Pisegna, Lyu et al. 2000). Upon stimulation, the parietal cell undergoes morphological changes, exposing the H,K-ATPase proton pump to the apical membrane, enabling acid secretion to take place (Forte, Machen et al. 1977).

Natural inhibitor of gastric acid secretion

Somatostatin is the natural inhibitor of gastric acid secretion. Somatostatin is produced by D-cells present in both the corpus and antrum. The antral D-cells are an “open enteroendocrine” cell type, with apical processes extending into the gastric lumen. They produce somatostatin in response to low luminal pH. However, the mechanism of pH sensing by D-cells in the antrum is not known. The D-cells in the corpus are “closed enteroendocrine” cell types and produce somatostatin in response to peptide and neuronal stimulation (Sandvik, Dimaline et al. 1993). Once produced, somatostatin binds to its receptor SSTR-2 on G-cells, ECL cells, and parietal cells. Somatostatin inhibits acid secretion directly on the parietal cell or indirectly by inhibiting gastrin and histamine release (Prinz, Sachs et al. 1994; Zaki, Harrington et al. 1996; Aurang, Wang et al. 1997).

Disorders of gastric acid secretion

Both overproduction and underproduction of acid have severe consequences. Large populations of people are affected by symptoms related to an increase in gastric acid secretion, varying from mild cases of heartburn and acid reflux disease to severe peptic ulcers. In contrast, a reduced level of acid secretion is mainly due to chronic atrophic gastritis that predisposes to gastric cancer.

Infection with *Helicobacter pylori* is one of the major causes of altered gastric acid secretion. The effect on acid secretion is dependent on the pattern

of gastritis. Antral gastritis stimulates acid secretion, whereas gastritis in the corpus results in low levels of acid production (McColl, el-Omar et al. 1998). Colonization with *Helicobacter* in the relatively high pH environment of the antrum triggers a Th1 predominant inflammatory response. The Th1 cytokine IFN-γ, produced in response to *Helicobacter* infection, not only induces the secretion of gastrin, but inhibits somatostatin production thus resulting in stimulated gastric acid secretion (Zavros, Rathinavelu et al. 2003). The role of acid inhibitory Th1 cytokines, including IL-1β and TNF-α, in antral gastritis has been established in vitro in which parietal cell acid secretion is inhibited. It is not known if these cytokines can inhibit acid secretion in the corpus, leading to an increase in pH and allowing the bacteria to spread and colonize the corpus. Chronic inflammation initiated by infection with *Helicobacter pylori* in the corpus induces gastric atrophy characterized by loss of acid secreting oxyntic glands. The mechanism by which inflammation triggers the loss of these oxyntopeptic lineages is not known. Recently, Shh was shown to be an important factor for gastric epithelial cell maturation and differentiation (van den Brink, Hardwick et al. 2002) . Loss of Shh expression has been correlated with gastric atrophy and intestinal metaplasia (van den Brink, Hardwick et al. 2002; Shiotani, Iishi et al. 2005; Shiotani, Iishi et al. 2006). Interestingly, in patients with chronic acid reflux resulting in fundic gland metaplasia of the esophagus, ectopic Shh expression was detected, which completely overlapped with expression of H,K-ATPase (van den Brink, Hardwick et al. 2002) suggesting that Shh signaling might promote the gastric epithelial phenotype.

Hedgehog signaling pathway

Vertebrate hedgehog genes were first reported a little over a decade ago (Echelard, Epstein et al. 1993; Krauss, Concordet et al. 1993; Riddle, Johnson et al. 1993; Chang, Lopez et al. 1994; Roelink, Augsburger et al. 1994). Three mammalian hedgehog genes have been identified, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). All three hedgehog genes are highly conserved between mouse and humans (Marigo, Roberts et al. 1995). Hedgehogs are produced as ~45 kDa precursor proteins that undergo a complex series of processing steps. First the 24 N-terminal amino acid residues comprising the signal peptide is removed, which is followed by autocatalytic cleavage to yield a 19kDa amino terminus protein and a 26 kDa carboxy terminal fragment. The carboxy terminus catalyzes the cleavage and acts as a cholesterol transferase (Lee, Ekker et al. 1994; Bumcrot, Takada et al. 1995).

Hedgehog signal reception and transduction

All three ligands can bind to the receptors Patched-1 (Ptch-1) and Patched-2 (Ptch-2). Hh ligands elicit their effects by antagonizing Patched activity. Once Hh binds Ptch, it relieves Ptch inhibition of a transmembrane protein called Smoothened (Smo). Once Smo is activated, it acts through a protein complex to block proteolytic processing of a zinc finger transcription factor family called Gli. The proteolyzed form of Gli is a potent transcriptional repressor, but upon stabilization by Hh signals full-length Gli translocates to the nucleus, where it binds and activates Hh target genes (Fig. 1.6). Three zinc

finger transcription factors exists Gli1, Gli2, and Gli3, which bind to the consensus sequence GACCACCCA in the promoter of Hh target genes(Kinzler and Vogelstein 1990; Ruppert, Vogelstein et al. 1990). Gli1 is completely dependent on Hh activity for its expression and primarily functions as a Hh activator (Marigo, Johnson et al. 1996; Lee, Platt et al. 1997; Platt, Michaud et al. 1997; Bai, Auerbach et al. 2002), while Gli2 exhibits both activator and repressor activities (Ruiz i Altaba 1999; Sasaki, Nishizaki et al. 1999). Gli3 is primarily a transcriptional repressor, although transcriptional activator properties have been described (Dai, Akimaru et al. 1999; Sasaki, Nishizaki et al. 1999; Shin, Kogerman et al. 1999).

Shh and its signaling in the embryonic stomach

The progenitors in the isthmus region in this regenerating tissue differentiate continually into specialized lineages. Yet, little is known about the mechanisms of their differentiation. Insights into the factors that regulate gut development and differentiation emerge from tissue recombination experiments (Mizuno, Yasugi et al. 1986; Hayashi, Yasugi et al. 1988) and gene targeting studies. Signaling molecules identified include hedgehog, transforming growth factor beta (TGF-β) and fibroblast growth factor (FGF) family members and their associated receptors (Kim, Hebrok et al. 2000; Ramalho-Santos, Melton et al. 2000; Aubin, Dery et al. 2002; Jacobsen, Narita et al. 2002; Kim, Huang et al. 2005; Spencer-Dene, Sala et al. 2006). These studies suggest a critical role for Shh in the proper patterning and development of gastric glands. Mice lacking Fgf10, Fgfr2b, activin receptor II or the transcription factor Gata4 ectopically

express Shh in the posterior stomach which results in simple glands and posterior expansion of the squamous epithelium (Kim, Hebrok et al. 2000; Jacobsen, Narita et al. 2002; Spencer-Dene, Sala et al. 2006). On the other hand, Shh null mice and Gli3 null mice display hyperplastic glandular mucosa with increased gland branching. The observed overgrowth of the mucosa was suggested to be due to a decrease in the rate of apoptosis (Ramalho-Santos, Melton et al. 2000; Kim, Huang et al. 2005). Therefore in a developing stomach, high levels of Shh seem to induce anterior gland formation while low levels seem to allow simple mucous gland formation but with marked hyperplasia in the corpus.

All of the reports mentioned thus far employed mice at different embryonic stages for their studies, partly, due to the fact that these genetically altered mice do not survive after birth. However, morphogenesis of the gland is not completed until the third week of postnatal life (Karam, Li et al. 1997). All the developmental pathways characteristic of adult epithelium were established postnatally (Karam, Li et al. 1997). Therefore, it might be important to establish the role of Shh and its signaling pathways using conditional tissue specific deletion and/ or targeted over expression.

Shh and its signaling in adult stomach

Most of the information about the functional role of Shh in an adult stomach is based on pathway inhibitor studies. Intriguingly, inhibition of hedgehog signaling using cyclopamine in vivo leads to enhanced proliferation

(van den Brink, Hardwick et al. 2001; El-Zaatari, Grabowska et al. 2008) whereas, cyclopamine on gastric cancer cell lines inhibit growth (Berman, Karhadkar et al. 2003; Ma, Chen et al. 2005; Yanai, Nagai et al. 2007) . An increase in cell proliferation upon cyclopamine treatment of primary mouse gastric cultures (Fukaya, Isohata et al. 2006) suggests, that the discrepancy between in vivo versus in vitro inhibition of Shh is not due to differences in the model systems. The increase in proliferation observed either in vivo or in primary cell culture suggests that the effects of hedgehog inhibition might be indirect. Indeed, cyclopamine treatment in vivo leads to hypergastrinemia (El-Zaatari, Grabowska et al. 2008), and gastrin is known to induce mucosal proliferation. However, it remains to be determined if hedgehog signaling directly effects gastrin gene expression or secretion.

Regulation of Shh in adult stomach

Not much is known about the extracellular signals regulating Shh gene expression in adult organisms. Yet recently, studies in the gastric cancer cell line 23132, and hypochlorhydric mouse models including H_2 receptor and gastrin null mice suggest a connection between the regulation of Shh expression and processing and gastric pH (Dimmler, Brabletz et al. 2003; Minegishi, Suzuki et al. 2007; Zavros, Waghray et al. 2007; El-Zaatari, Grabowska et al. 2008). Loss of Shh expression during *Helicobacter*-induced inflammation and its re-expression after eradication (Nishizawa, Suzuki et al. 2007; Shiotani, Uedo et al. 2007) suggests a role for inflammation in regulating Shh expression. Indeed, IFNγ has been shown to regulate Shh gene expression in medulloblastomas during the

initiation of neoplastic transformation (Wang, Pham-Mitchell et al. 2003; Lin, Kemper et al. 2004; Wang, Lin et al. 2004). However, the mechanism by which inflammatory cytokines regulate Shh expression remains unknown.

Overview of thesis

Aberrant activation of Shh and its signaling pathway is associated with tumorigenesis in various gastrointestinal tissues including stomach (Berman, Karhadkar et al. 2003). While hedgehog (Hh) signaling in the normal adult stomach appears to be both paracrine and autocrine (van den Brink, Hardwick et al. 2002; Stepan, Ramamoorthy et al. 2005; Fukaya, Isohata et al. 2006), gastric cancer cells seem to autonomously regulate their proliferation, promoting tumor growth in an autocrine manner (Berman, Karhadkar et al. 2003). Moreover, it has been proposed that reducing the levels of soluble Hh ligand may be effective in shrinking tumors (Berman, Karhadkar et al. 2003). While the pharmaceutical industry has spent many years and large amounts of money developing small molecule inhibitors of this pathway to treat the disease, precise mechanisms involved in the regulation of Shh and its role in gastric physiology and pathophysiology are not yet completely understood. There is still considerable confusion about the source and direction of Hh signaling in stomach; it is not yet known whether the Hh signal is paracrine from epithelium to mesenchyme, or whether the epithelium is competent to receive an autocrine Hh signal. I have addressed this question using reporter mice for Shh and its target genes in Chapter II of my dissertation.

Gastric cancer is a multistage disease process. According to Correa's model of human gastric neoplasia (Correa, Haenszel et al. 1975), development of chronic inflammation or gastritis is the initial trigger initiating the disease process. While inflammation of the stomach can result from various factors, infection with *Helicobacter* seems to be the most important risk factor for cancer development. Chronic inflammation then leads to atrophy, progressing to metaplasia, dysplasia and cancer.

Loss of Shh expression has been correlated with gastric atrophy and metaplasia in patients infected with *Helicobacter pylori* (Shiotani, Iishi et al. 2005; Shiotani, Iishi et al. 2006; Nishizawa, Suzuki et al. 2007; Shiotani, Uedo et al. 2007). However, it is not known whether loss of Shh expression triggers these events, or if gastric atrophy (loss of parietal and or zymogenic cells) precedes the observed loss in Shh expression. I have addressed this question in Chapter III of my dissertation using *Helicobacter*- infected Shh reporter mice.

Finally, the concluding chapter (chapter IV) summarizes my findings. Implications of these findings and suggestions for further investigations are presented.

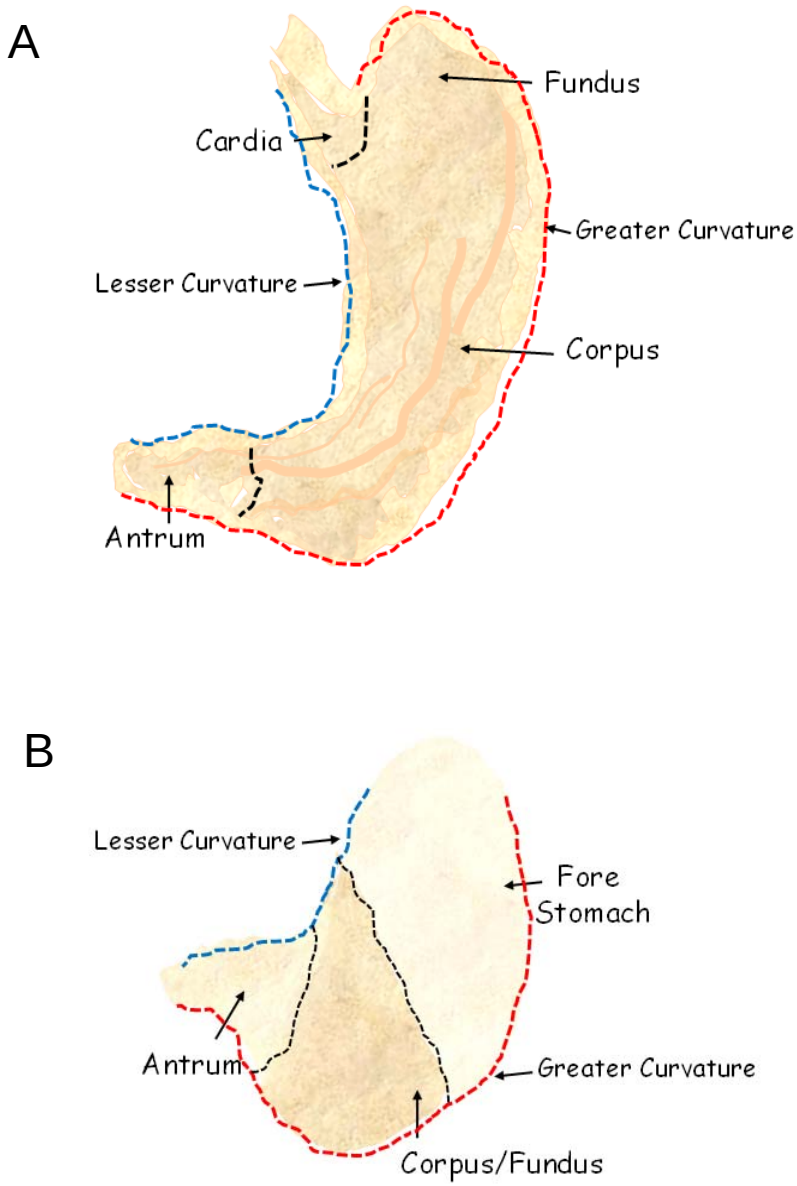

Figure 1.1: Human and mouse stomach

A sketch showing specific regions of the stomach, and variations between human (A) and mouse stomach (B). The human stomach is comprised of 4 regions: cardia, fundus, corpus (body) and antrum. However, the mouse stomach is comprised of a forestomach lined by squamous epithelium (like the esophagus), as well as corpus/ fundus and antrum

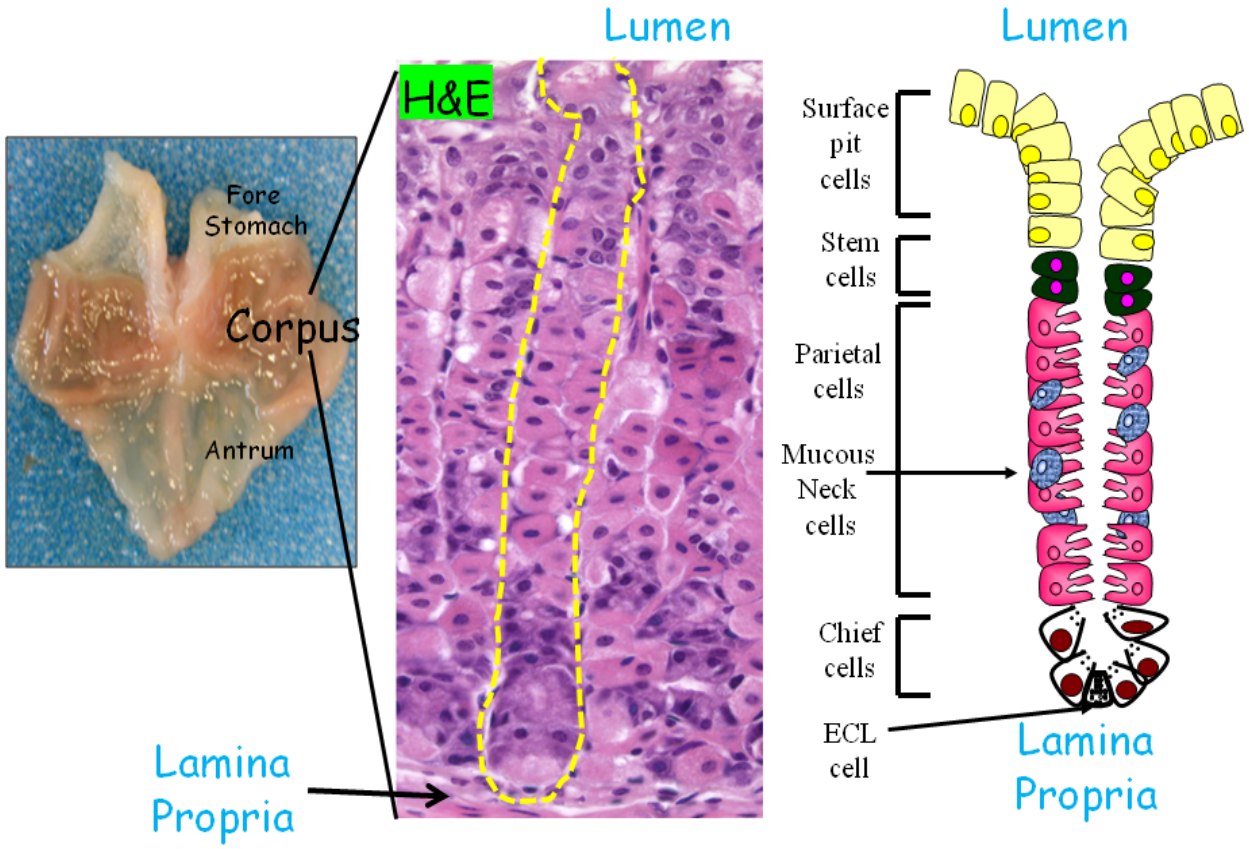

Figure 1.2: Anatomy of the corpus gastric gland

The different regions of the stomach, forestomach, corpus and antrum are distinguishable macroscopically. Single layer of columnar epithelium invaginates into lamina propria producing blind tubular units called glands. The gland is divided into four regions: surface pit, isthmus, neck and base. The pluripotent stem cell and the progenitor cells reside in the isthmus region. The pit precursor and the pit cells are located near the surface towards the lumen, the mucous neck cells in the neck region, pre-zymogenic and zymogenic cells along the base and parietal cells in the surface, neck and base region of the gastric unit.

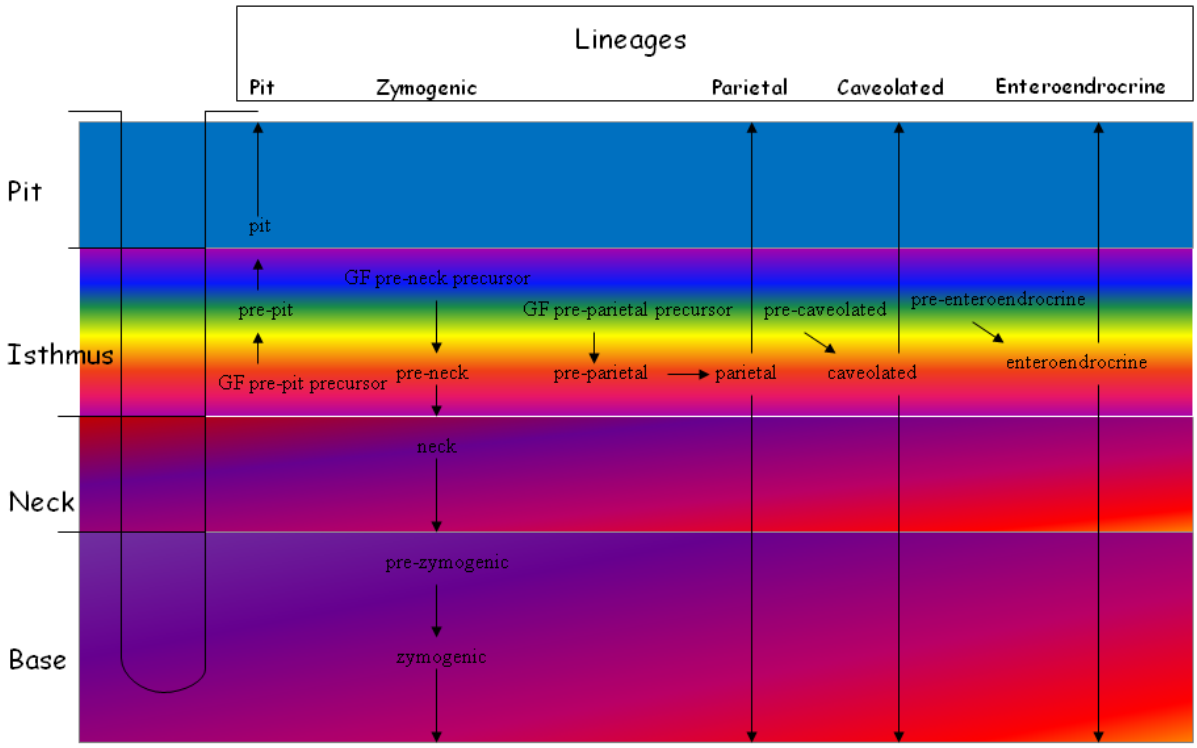

Figure 1.3: Summary of gastric epithelial differentiation within the corpus
The pluripotent stem cell in the isthmus gives rise to three different types of progenitor cells pre-pit, pre-neck, and pre-parietal cells. The pre-pit cells migrate towards the lumen, the pre-neck towards the base. The pre-parietal cells, entero-endocrine cells, and caveolated cells originate from the isthmus then migrate both in an outward and inward direction. Different colors represent the different regions and the shading demonstrates the pattern of differentiation. This figure has been modified from (Karam and Leblond 1993).

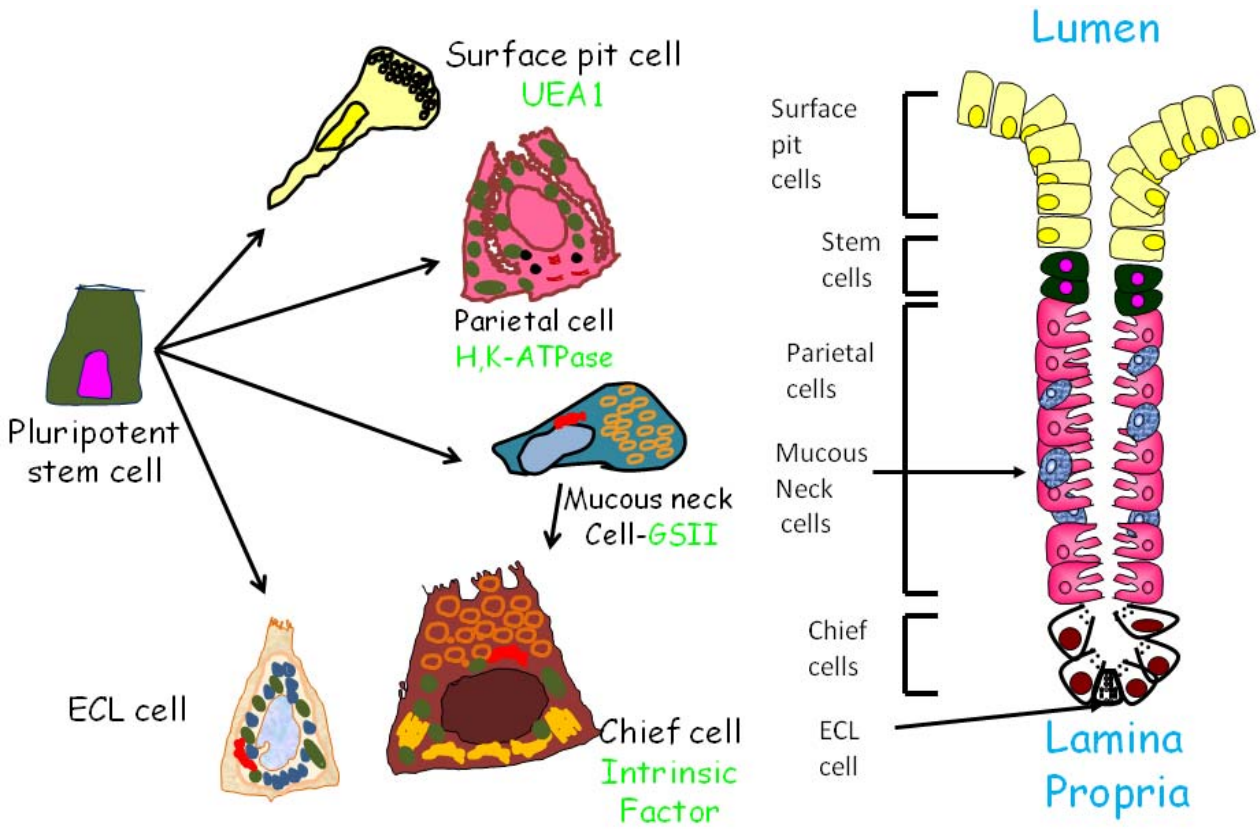

Figure1.4: Model of differentiation in the corpus gland

The pluripotent stem cell gives rise to surface pit cells identified by UEA1 lectin, parietal cells marked by H, K-ATPase, neck cells identified by GSII lectin, and ECL cells. Neck cells transform into pre-zymogenic cells and then differentiate into chief cells identified by intrinsic factor. These cells originating from the isthmus then migrate in an outward and /or inward direction to occupy the length of the gland

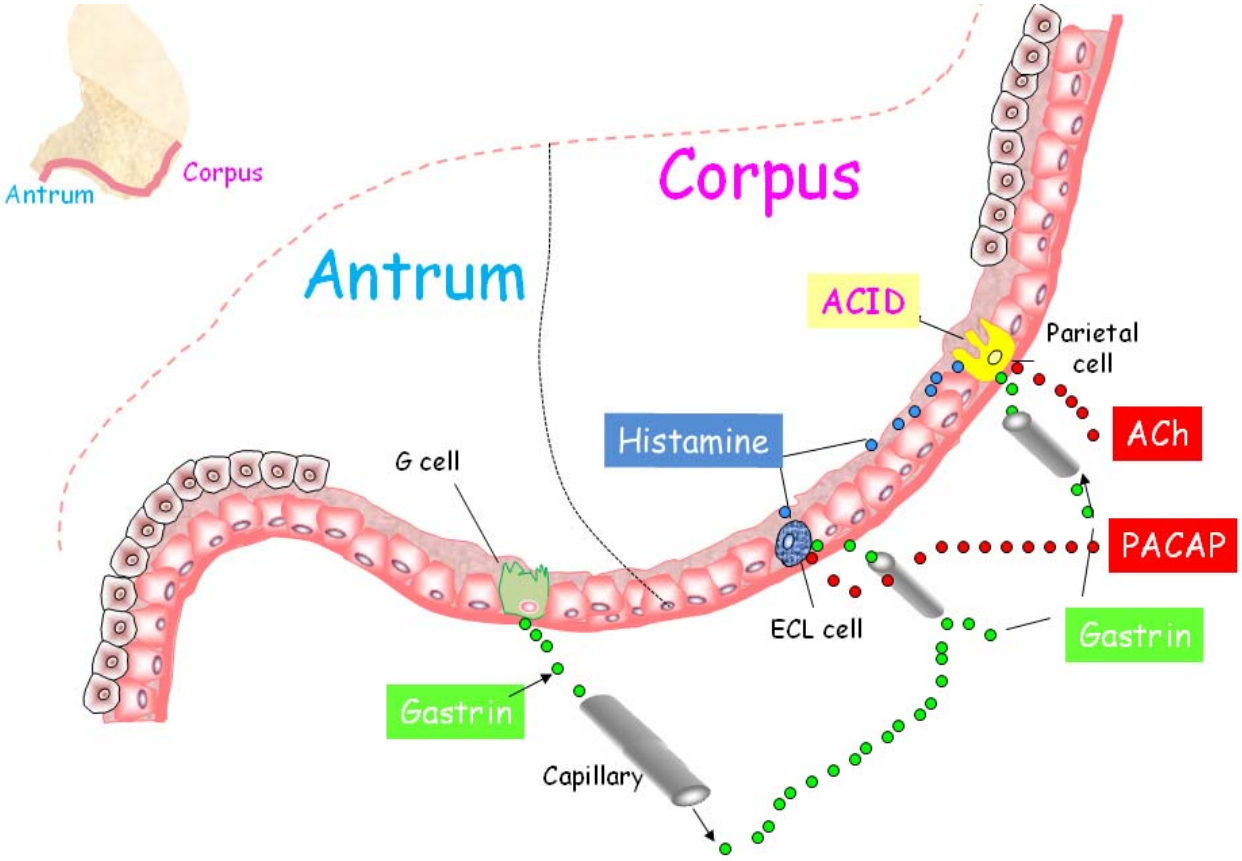

Figure 1.5: Physiology of gastric acid secretion

Parietal cells are stimulated by three main agonists' gastrin, histamine, and acetylcholine (Ach). Gastrin is secreted from G-cells in the antrum into the blood and stimulates ECL cells and parietal cells. Vagal terminals release Ach and PACAP neurotransmitters. Ach stimulates parietal cells, whereas, the PACAP stimulates both ECL and parietal cell. Histamine is released from the ECL cell in response to these stimuli and along with gastrin and Ach stimulates acid secretion form the parietal cells.

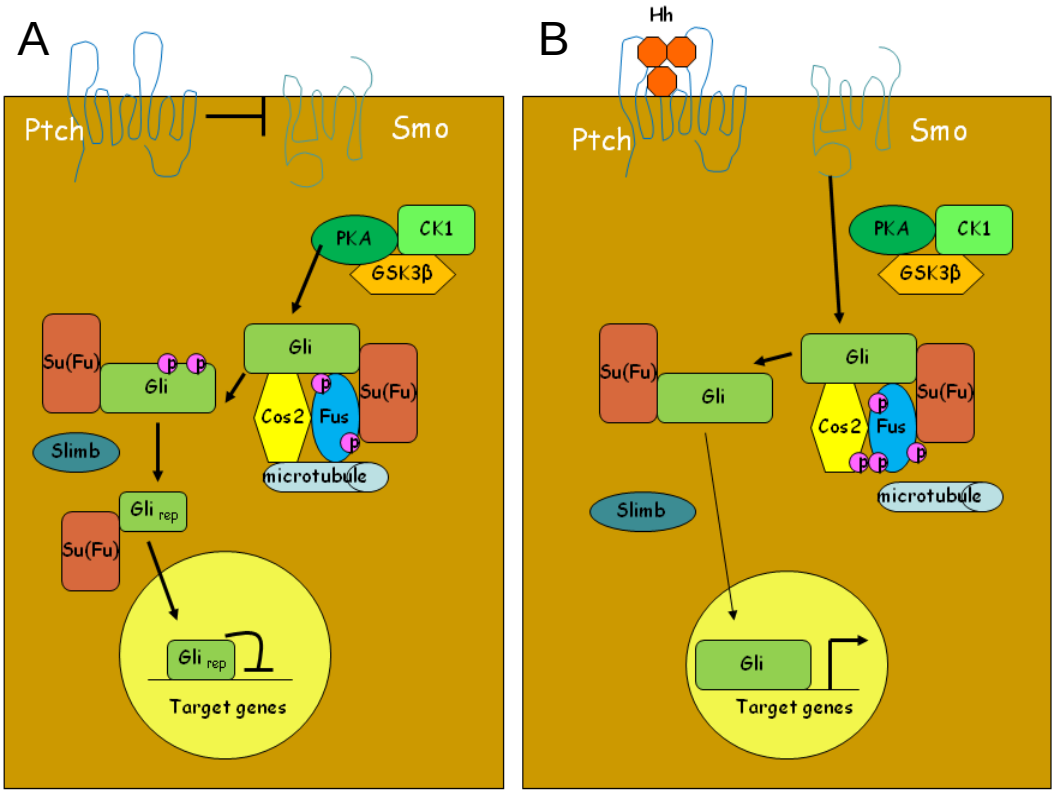

Figure 1.6: Hedgehog (Hh) signal transduction.

A. In the absence of Hh signal, Patched inhibits the positive signal of smoothened. A multiprotein complex, consisting of the Zinc-finger transcription factor Gli1, Costal2 (Cos2), Fused (Fu), and Suppressor of fused (Su (Fu)), associates with microtubules. Through phosphorylation by PKA, CK1 and GSK3-β, Gli is targeted for proteolytic cleavage via an F-box protein (Slimb) into a repressor isoform (Gli_{rep}) that acts to repress target genes in the nucleus. B. Upon Hh binding to Ptch, Smo inhibition is relieved, Cos2 and Fu are recruited to Smo, and Fu acts to repress Su (Fu). This causes hyperphosphorylation of Fu and Cos2, and subsequent dissociation from microtubules. Full length Gli is then stabilized and acts in the nucleus by binding promoters of specific target genes and activating their expression.

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CHAPTER II

SONIC HEDGEHOG EXPRESSION AND PARACRINE SIGNALING IN THE ADULT MOUSE STOMACH

ABSTRACT

Background & Aims: The Sonic hedgehog pathway regulates normal gastric morphogenesis. Mice deficient in hedgehog pathway components exhibit abnormal growth of the gastric mucosa. However, there is conflicting information regarding the region and the cell types expressing and responding to Shh. We determined the patterns of Shh expression and responses in the adult mouse stomach. Methods: $Shh^{LacZ/LacZ}$, $Ptch1^{LacZ/+,}$ and $Gli1^{LacZ/+}$ reporter mice were used to determine the expression of Shh and its signaling targets Ptch1 and Gli1 using β-galactosidase histochemistry. RT-PCR analysis of endogenous gene expression in isolated epithelium and mesenchyme was performed to confirm the X-gal staining patterns. Primary cultures of cells from $Gli1^{LacZ/+}$ mice were treated with Shh to assess the presence of autocrine signaling. Results: Not all parietal cells express Shh. Shh was expressed primarily in parietal cells of the neck/isthmus; while expression decreased towards the gland base. In contrast to prior reports, we found that antral cells express Shh. Also we here report for the first time that Gli1expression in the adult mouse stomach is confined to the

mesenchyme. Conclusion: Hh signaling in the gastric corpus and antrum is paracrine.

INTRODUCTION

Shh null mice and Gli3 null mice display hyperplastic glandular mucosa with increased gland branching (Ramalho-Santos, Melton et al. 2000; Kim, Huang et al. 2005). However, it is not known if the phenotype seen in the Shh null mice results from autocrine signaling, or paracrine signaling from the neighboring epithelial or mesenchymal cells. Several studies have documented the expression of Shh in the gastric corpus (van den Brink, Hardwick et al. 2001; van den Brink, Hardwick et al. 2002; Fukaya, Isohata et al. 2006; El-Zaatari, Tobias et al. 2007; Minegishi, Suzuki et al. 2007). However there is conflicting information regarding which cell types actually express Shh. In early studies, Shh protein expression in the mouse stomach was observed in the mucous neck, parietal and chief cells (van den Brink, Hardwick et al. 2001; van den Brink, Hardwick et al. 2002) while Fukaya, et al. reported expression only in the parietal cells (Fukaya, Isohata et al. 2006). Employing in-situ hybridization and immunostaining techniques, El-Zaatari et al. reported that Shh mRNA and protein expression is located in surface pit and parietal cells (El-Zaatari, Tobias et al. 2007). Subsequently, Minegishi Y. et al. reported that Shh mRNA and protein is expressed in the basal glandular region including parietal and chief cells (Minegishi, Suzuki et al. 2007). Determining the expression pattern is critical to understanding Hh signaling in this organ.

To date, no genetic models have been used to study the expression of Shh and its signaling components in the adult stomach. We used the Shh-LacZ reporter mice to determine the cellular origin of Shh expression. To identify the

cells capable of responding to Shh, expression of Ptch1 and Gli1 was examined using the Ptch1$^{lacZ/+}$ and Gli1$^{lacZ/+}$ reporter mice respectively. Ptch1 is both a Shh receptor and a transcriptional target for Hh signaling (Goodrich, Johnson et al. 1996; Marigo, Davey et al. 1996; Stone, Hynes et al. 1996; Agren, Kogerman et al. 2004; Nagao, Toyoda et al. 2005), but the expression of Ptch1 is not solely dependent on Hh (Borjigin, Deng et al. 1999; Zhang, Zhao et al. 1999; Long, Schipani et al. 2001). Gli1 is a direct target of Hh and its expression is highly dependent upon active Hh signaling (Bai, Auerbach et al. 2002). Regional expression of Shh and its signaling molecules (Ptch1 and Gli1) were confirmed by performing RT-PCR analysis on the enriched/separated epithelial and mesenchymal fractions.

MATERIALS AND METHODS

Mice

Shh $^{LacZ/LacZ}$, Ptch1$^{lacZ/+}$, and Gli1$^{lacZ/+}$ mice have been described elsewhere (Goodrich, Milenkovic et al. 1997; Park, Bai et al. 2000; Jeong, Mao et al. 2004). The Shh $^{LacZ/LacZ}$ mice were maintained as a homozygous colony and the Ptch1$^{lacZ/+}$ and Gli1$^{lacZ/+}$ colonies were maintained in the heterozygous state and genotyping was performed according to the published protocol (Goodrich, Milenkovic et al. 1997; Bai and Joyner 2001; Bai, Auerbach et al. 2002). The study was performed with the approval of University of Michigan Animal Care and Use Committee, which maintains an American Association for Assessment and Accreditation of Laboratory Animal Care facility.

X-gal staining

The stomach was opened along the greater curvature and the gastric contents were washed in ice cold PBS. The stomach was fixed in fresh 4% paraformaldehyde/PBS (pH 7.0-7.5) for 1h at 4^{o}C. The stomach was washed three times for 30 min each with β-gal rinse buffer (100 mM sodium phosphate pH 7.3, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40) at room temperature. The tissue was incubated for 16h at 37^{o}C in β-gal staining solution (β-gal rinse buffer, 25 mg/ml X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide). The tissue was rinsed with β-gal rinse buffer for 30 min

at room temperature, and post-fixed overnight in 10% paraformaldehyde/PBS. The tissue was processed and paraffin-embedded prior to sectioning.

mRNA analysis

Resected tissue was collected in Trizol reagent (Invitrogen) total RNA was extracted, purified, and DNase treated using the RNeasy kit (Qiagen). Using the iScript cDNA synthesis kit (BioRad), cDNA was synthesized from 1μg of total RNA. QRT-PCR was performed using a BioRad I cycler with SYBR Green dye (Molecular Probes). Each 20 μl reaction contained 2μl of reverse-transcribed product, 1x PCR buffer, $MgCl_2$, 100nM of each primer, 1x SYBR Green, 10nM fluorescein, 200mM dNTPs, and 0.025U of Platinum Taq polymerase (Invitrogen). Each PCR amplification was performed in triplicate wells with the following conditions: 3 min at 95^{o}C, 40 cycles of 9s at 95^{o}C and 1min at 60^{o}C, followed by 1min at 55^{o}C. Melt curve analysis was used to assess the purity of the product. Beacon software (BioRad) was used to design primer sequences Shh reverse ATCGTTCGGAGTTTCTTGTGAT, Shh forward ATGTTTTCTGGTGATCCTTGCT, GAPDH reverse TAT TAT GGG GGT CTG GGATGG, GAPDH forward TCA AGA AGG TGG TGA AGC AGG. Data for Shh were normalized to the expression of GAPDH.

Immunoblot analysis

Protein was loaded on a 4-20% SDS-PAGE gradient gel for immunoblot analysis. The membranes (Hybond-C Extra Nitrocellulose, Amersham Biosciences) were blocked with Detector Block (KPL, Gaithersburg, MD, 71-83-

00) for 1h at room temperature followed by an overnight incubation with a 1:200 dilution of the goat polyclonal anti-Shh (Santa Cruz Biotechnology, sc-1194), or a 1h incubation with1:5000 GAPDH (Chemicon) antibody. The membranes were washed twice for 10 min each in 1x TBST and incubated for 1h with a 1:5000 dilution of horseradish peroxidase-conjugated secondary anti-goat, or anti-mouse antibodies. The membranes were washed five times for 30 min with 1x TBST. Proteins were visualized using enhanced chemiluminescence (Lumilight substrate, Roche Applied Science, Mannheim, Germany).

Epithelial-mesenchymal separation of the adult stomach

Stomachs from adult C57BL/6 mice were opened along the greater curvature and rinsed in ice cold saline to wash out the contents. The fore stomach was discarded and under a dissecting microscope, the corpus and antrum were separated. A modified version of the Dual- buffer method was used to separate epithelium and mesenchyme (Merchant and Heller 1977). Each of these sections were rinsed in 0.154 M NaCl-1mM DTT solution. Individual sections were incubated in Solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, in 1000 ml H_2O, pH 7.3) for 15 min at 37^{o}C. The solution was discarded and the individual sections were now incubated in solution B (1.5mM EDTA and 0.5mM DTT in 1000ml PBS with Ca^{+2} and Mg^{+2}) for 3-4h. The supernatant was collected as the epithelial fraction and the pellet as the mesenchymal fraction. Each of these fractions was centrifuged at 900xg for 5 min and washed twice before using for RNA isolation. The pellets were homogenized in 1ml TRIzol (invitrogen) to prepare RNA. Using iScript

cDNA synthesis kit (Bio-Rad), cDNA was synthesized from 1μg total epithelial or mesenchymal RNA. RT-PCR was performed for 30 cycles using the primer sets shown in Table1 to detect expressed genes.

Immunohistochemical Staining

X-gal stained tissue sections were deparaffinized and rehydrated. The sections were then washed with 1x PBS, blocked with 20% serum and incubated with a 1: 50 dilution of HRP conjugated anti-GSII , a 1:500 dilution of rabbit anti-Intrinsic factor or a 1:125 dilution of mouse anti-H,K-ATPase –β subunit (Medical & Biological Laboratories Co., LTD) for 1h. Staining was visualized with avidin-biotin complexes by using the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) using liquid diaminobenzidine for the substrate (DAB, DAKO). H, K-ATPase staining was performed using the Ark-labeling kit (Dako).

Immunofluorescence

Immunofluorescence staining was performed on 5 μm paraffin sections and on mouse primary cultured cells. Tissue sections were deparaffinized, rehydrated and blocked with 20% serum. The primary cultured cells were fixed, permeablized, stained with X-gal then blocked with 20% serum. A 1:500 dilution of rabbit anti-UEA1 (Sigma), and a 1:800 dilution of mouse anti-H,K-ATPase (Medical & Biological Laboratories Co., LTD) antibodies were used on X-gal stained sections and X-gal stained cells respectively, followed by a 1:500 dilution of fluorophore–labelled Alexafluor secondary antibody (Molecular probes). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, 1 μg/ml,

Sigma) for tissue sections and images were generated on a Nikon Eclipse E800 microscope (Nikon, Melville, NY) using a SpotCD camera.

Mouse primary cell culture and Shh treatment

Mouse primary cells were isolated according to the previously published protocol (Hinkle, Bane et al. 2003). Briefly, stomachs from $Gli1^{lacZ/+}$ mice were opened along the greater curvature. Corpus was isolated from individual mice, and epithelial cells were dispersed with pronase, centrifuged and the pellet resuspended in 10% RPMI. The cells from individual stomachs were seeded on Matrigel (BD Biosciences) coated chamber slides. Fifty micro liters of Matrigel was added in the slide chamber and the slide was incubated for 20-30 min prior to seeding the cells. The cells were treated with 1 μg/ml mouse recombinant Shh (R&D systems) for 24h.

RESULTS

Shh is predominantly expressed in the corpus

Conclusions from in situ hybridization and immunohistochemistry analyses determining the cellular origin of Shh expression in the stomach have been conflicting. The recent development of Shh-LacZ reporter mice (Jeong, Mao et al. 2004) provided us with a novel tool to study the expression of Shh in vivo. In the Shh-LacZ mice, the *E. coli* LacZ gene containing a SV-40 nuclear localization signal was inserted into the 3'UTR of the Shh gene locus downstream of IRES (Fig. 2.1A). Whole mounts of stomach tissue from non transgenic (Wt) and transgenic Shh-LacZ reporter mice were incubated with β-galactosidase substrate (X-gal). We observed that Shh expression was highest in the corpus, low in the antrum and undetectable in the forestomach (Fig. 2.1B). Also there appeared to be modest Shh expression in Brunner's glands, though some background LacZ staining was observed in the non-transgenic mouse stained simultaneously (Fig. 2.1B). Since the LacZ gene was inserted into the 3' UTR of the mouse Shh locus, quantitative analysis of Shh expression was performed on tissue extracts from the corpuses of wild type and Shh LacZ reporter mice. Similar levels of Shh mRNA and protein were found in both the Wt and Shh LacZ reporter mice demonstrating that the reporter gene did not alter endogenous Shh expression (Fig. 2.1C, D).

Shh is exclusively expressed in the epithelium

We used several approaches to clearly establish the expression of Shh in the normal adult stomach. We examined the expression of Shh using Shh-LacZ reporter mice. In the corpus, LacZ staining was seen in all major epithelial cells (Fig. 2.2A). The corpus glands closest to the forestomach consistently showed stronger Shh expression (Fig. 2.2A). We demonstrated that all major cell types in the corpus express Shh (Fig. 2.3) by co-localizing LacZ expression with surface pit (UEA1) (Fig. 2.3A), mucous neck (GSII) (Fig. 2.3B), parietal (HK-ATPase) (Fig. 2.3C) and chief (intrinsic factor) (Fig. 2.3D) cell markers. Of note, some parietal cells do not stain for LacZ. The location of the parietal cells not expressing Shh is primarily but not exclusively in the lower glandular region. In the antrum, we detected expression in the glandular epithelium (Fig. 2.2B). The β-gal staining was mostly seen in the surface mucous cells of the antral glands (Fig. 2.2B). Stromal cells in both corpus and antrum were completely negative for Shh expression (Fig. 2.2A, B).

To confirm the location of Shh expression, the epithelial and mesenchymal components of adult stomach were separated and each fraction was assayed for specific genes known to be expressed in the epithelium (HK-ATPase and Gastrin) or mesenchyme (Actg2) by RT-PCR (Fig. 2.2C, D). Shh expression was detected in both corpus and antrum and was found to be restricted to the epithelium (Fig. 2.2C, D). Thus the combined evidence strongly indicates that Shh is expressed exclusively in the epithelium.

Epithelial expression of Ptch1 in the corpus

The expression of Ptch1 was examined by X-gal staining of $Ptch^{LacZ/+}$ mice (Fig 2.4A). In the corpus, LacZ staining was seen in both epithelial and mesenchymal regions (Fig. 2.4A). In the antrum, the staining was restricted to the mesenchymal compartment. To confirm Ptch1 expression, the epithelial and mesenchymal components of the adult stomach were separated (Fig. 2.4B) and the enriched fraction was assayed by RT-PCR for specific genes known to be expressed in the mesenchyme (Actg2) or both tissue layers (HPRT) (Fig. 2.4B). RT-PCR was then performed on each fraction to detect Ptch1 expression. Ptch1 expression was detected in both the epithelium and mesenchyme of the corpus but was strictly mesenchymal in the antrum (Fig. 2.4B).

Gli1 is exclusively expressed in the mesenchyme

To determine if Hh signaling in the adult stomach is autocrine, as suggested by earlier reports (van den Brink, Hardwick et al. 2001; Stepan, Ramamoorthy et al. 2005; Fukaya, Isohata et al. 2006) or paracrine, we used the $Gli1^{LacZ/+}$ reporter mice. X-gal staining in the $Gli1^{lacZ/+}$mice identified the cells that actively respond to Hh signals. Nuclear β-galactosidase staining was observed in the mesenchymal compartment of both the corpus and antrum (Fig. 2.5A). Despite careful examination of several sections from several mice, no staining was seen in the epithelial compartment of either corpus or antrum. The expression of Gli1 in the mesenchymal compartment was confirmed by RT-PCR analysis for Gli1 and Gli2 after non- enzymatic separation of the epithelium and

mesenchyme. The RT-PCR confirmed that the expression of Gli1 is strictly mesenchymal (Fig. 2.5B).

This is in contrast to reports in vitro, demonstrating autocrine Hh signaling in gastric primary and transformed cells upon stimulation with exogenous Shh (Berman, Karhadkar et al. 2003; Stepan, Ramamoorthy et al. 2005). To test this possibility of autocrine Shh signaling, primary cells from $Gli1^{lacZ/+}$ mice were isolated and treated with Shh for 24h. Cells were stained with X-gal to detect Gli1 expression (Fig. 2.6A, D) and co-stained with the parietal cell marker H, K-ATPase (Fig. 2.6B, E). Gli1 reporter expression was observed in the cells expressing H, K-ATPase (Fig. 2.6C, F) only upon stimulation with Shh. In the untreated cells, LacZ staining was observed only in the spindle shaped cells and not in the parietal cells (Fig. 2.6 G H). Despite the finding that parietal cells can express Gli1 in vitro, we failed to find any parietal cell expressing Gli1 in a normal adult stomach emphasizing the significance of in vivo studies.

DISCUSSION

The expression of Shh and Ptch1 in the adult stomach was first reported in 2001(van den Brink, Hardwick et al. 2001). Since then, several reports have been published documenting the expression of Shh in the adult stomach. Nearly a decade later, there is still no clear understanding of the cells that express or respond to Shh in the adult stomach. Therefore, there is a pressing need for a careful study to analyze and characterize the expression of Shh and its signaling pathway in the adult stomach. Most of the reports based their conclusions regarding the expression of Shh on immunostaining using the same antibody, Shh-N-19 (Santa Cruz). This antibody was raised against the highly conserved N-terminal region and hence can cross react with both Ihh and Dhh. Therefore, in this study, reporter mice were used to validate Shh expression and signaling.

Although Shh LacZ reporter mice were used, certain caveats must be considered. First, these mice show the expression of Shh mRNA and not protein. Therefore the detection of X-gal in a cell indicates gene expression. Whether the protein is produced or whether it is biologically active cannot be determined using the Shh LacZ mice alone. Rather, LacZ expression must be coupled to the expression of target genes (e.g. Gli1). Second, the LacZ stability might be different from that of Shh. Nonetheless, using this mouse model, we can determine specific Shh expression independent of other hedgehog family members e.g., Ihh and Dhh.

We noted that the corpus glands near the corpus-forestomach junction, and the surface pit cells were the earliest to stain for X-gal. It remains to be determined if these glands or cells express high levels of Shh or just stably express X-gal. The relevance of putative higher levels of Shh in this region of stomach is related to the recent rise in incidence and prevalence of the adenocarcinoma of the esophagogastric junction in the Western population. We also noticed, in progressing from the proximal to distal stomach, that high X-gal staining was observed predominantly in the upper 1/3rd of the glands closest to the forestomach, but became predominant in the lower glandular region closer to the transition zone. Thus, one explanation for differences in detection of Shh expression might be due to differences in the regions of the stomach examined.

In contrast to earlier studies reporting an absence of Shh expression in the antrum (van den Brink, Hardwick et al. 2002), we detected X-gal staining in the antrum. The expression was not as robust as that seen in the corpus. The expression was patchy and was mostly seen in the surface mucous cells.

We noted that not every parietal cell expressed Shh. This was not the case with other cell types in the corpus. Shh expression was primarily absent in the parietal cells closest to the base of the gland. The parietal cells at the base of the gland are more mature parietal cells destined for destruction (Karam, Yao et al. 1997). These parietal cells, which are farthest away from the lumen, are not involved in acid secretion (Karam, Yao et al. 1997).

Lack of Shh expression from these mature parietal cells could indicate that the parietal cell at the base of the gland has gland has moved away from an inductive signal or from “a regulatory cell” located at the isthmus. If this were the case, further investigations might lead to important findings regarding regulation and or function of Shh in the stomach.

Attempts have been made to understand the functional role of Shh in the stomach. However, since the Shh null mice die at or soon after birth the data is mostly based on pharmacological inhibition of Hh pathway by cyclopamine or in vitro studies (van den Brink, Hardwick et al. 2001; van den Brink, Hardwick et al. 2002; Stepan, Ramamoorthy et al. 2005; Fukaya, Isohata et al. 2006). Not much has been reported about the cellular targets of Shh in the adult stomach. Ptch1, which is both Hh receptor and target gene has been the only marker used to identify the cells responding to Hh signaling in the stomach. The expression of Ptch1 is shown in parietal, chief and mesenchymal cells in the corpus (van den Brink, Hardwick et al. 2001) . A recent finding suggests Ptch1 expression is not solely dependent on Hh signaling (Borjigin, Deng et al. 1999; Zhang, Zhao et al. 1999; Long, Schipani et al. 2001). Gli1 is considered a direct target of Hh and its expression is highly dependent upon active Hh signaling (Bai, Auerbach et al. 2002). The only Gli1 expression reported thus far in the mouse stomach is at E18.5 where the expression was shown to be mesenchymal (Ramalho-Santos, Melton et al. 2000).

One study raised the possibility of autocrine Shh signaling. Increase in H, K-ATPase expression following Shh treatment was shown in canine primary

parietal cells (Stepan, Ramamoorthy et al. 2005). We tested this in our own hands using the Gli1$^{lacZ/+}$ mice and found that the possibility exists for autocrine signaling upon stimulation of cells in vitro with Shh. However, none of our attempts to find this in vivo were successful. Therefore we concluded that Gli1 expression is strictly mesenchymal in adult mouse stomach.

The understanding in the field about the Hh signaling in an adult mouse stomach thus far is solely based on Ptch1 expression. According to the current understanding, Hh signaling is both autocrine and paracrine in the adult stomach. Our data, at least for mouse, contradict this. We have documented the expression of Gli1 strictly in the mesenchyme of an adult mouse stomach, which suggests Hh signaling is paracrine.

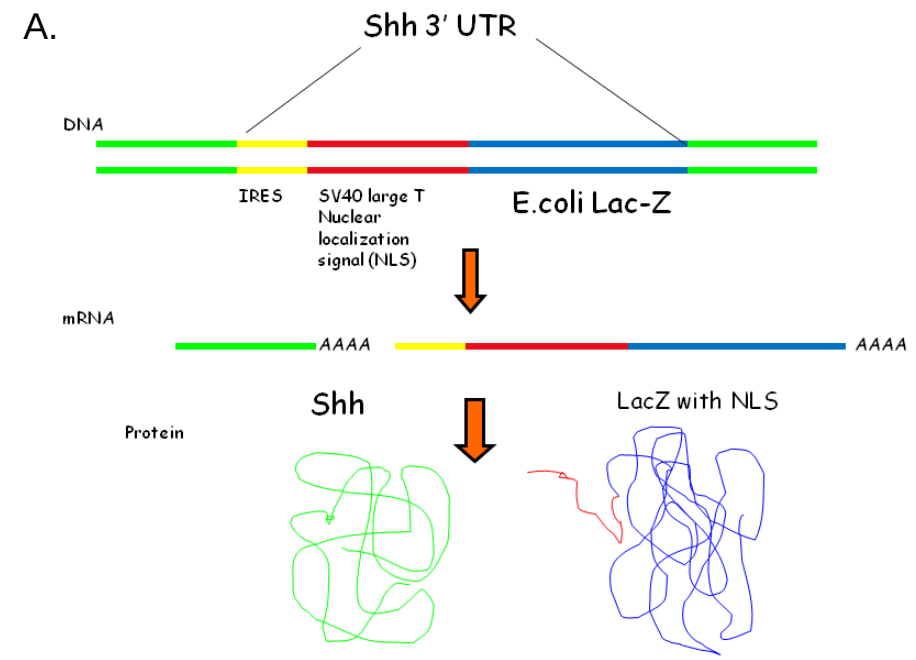
In conclusion, I have demonstrated that Shh expression is seen in the corpus and antrum of the adult mouse stomach. The expression of Shh is strictly epithelial and is found in all major epithelial cell types including surface pit, mucous neck, parietal and chief cells, although not all parietal cells express Shh. The expression of Ptch1 was observed in both epithelial and mesenchymal compartments of the corpus but only in the mesenchymal compartment in the antrum. The Hh target and signaling molecule Gli1 is expressed strictly in mesenchyme in the adult stomach. Thus, it is likely that canonical Hh signaling is paracrine in normal adult mouse stomach.

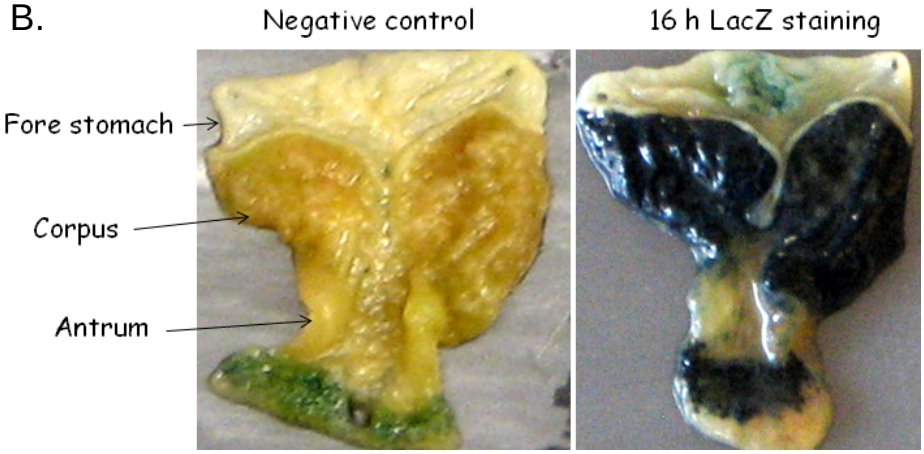
Gene	Forward Primer	Reverse Primer	PCR product
HPRT	5'-AGTCCCAGCGTCGTGATTAGC-3'	5'-ATAGCCCCCCTTGAGCACACAG-3'	204bp
H,K-ATPase	5'- AAGTCACCAGGGGTAACCTTGAG -3'	5'- TGTGCTTTCTTGCCATAGTAGGG -3'	491bp
$Actg_2$	5'- GGGTGTGATGGTGGGAATGG -3'	5'- GGTGCTCTTCTGGTGCTACTCG -3'	182bp
Shh	5'-GCTGTGGAAGCAGGTTTCG-3'	5'-GGAAGGTGAGGAAGTCGCTG-3'	232bp
Ptch1	5'-TGTTGGTGTGGATGATGTC-3'	5'-TGTGAGGCTCTGTGTAGG-3'	377bp
Gli1	5'-CGCCAAGCACCAGAATCGG-3'	5'- CCGACAGAGAAGGTCCTTCATCC-3'	188bp
Gli2	5'-CTCCAACCTTCTGTCATC-3'	5'-TAGTAGTATAGCGTGTTCTG-3'	297bp

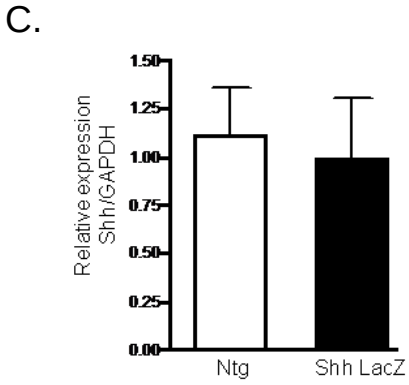
Table 2.1 RT-PCR primers

Figure 2.1: Shh is predominantly expressed in the corpus

Schematic representation of Shh targeted locus (A). *E.coli* LacZ gene with SV40 nuclear localization signal and IRES was inserted in the 3'UTR of the Shh gene locus. The LacZ was driven by the Shh promoter. Whole mounts of stomachs from a nontransgenic (NTg) and a Shh-LacZ reporter mouse (B) are shown after incubating the tissue in a β-galactosidase substrate (X-gal) for 16h. Quantitative RT-PCR was performed on corpus RNA from nontransgenic and Shh LacZ reporter mice. Shown is the ratio of Shh to GAPDH mRNA (C). The mean ± SEM for three mice is shown. An immunoblot of protein isolated from the corpus of nontransgenic and Shh-LacZ mice is shown in D, the immunoblot for Shh was re-blotted for GAPDH. Protein expression was quantified using the image-J software (NIH). The mean ± SEM for Shh/GAPDH is shown (D).


A.
Shh 3' UTR
DNA
IRES
SV40 large T Nuclear localization signal (NLS)
E.coli Lac-Z
mRNA
AAAA
AAAA
Protein
Shh
LacZ with NLS


B.
Negative control
16 h LacZ staining
Fore stomach
Corpus
Antrum


C.
Relative expression Shh/GAPDH
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Ntg
Shh LacZ

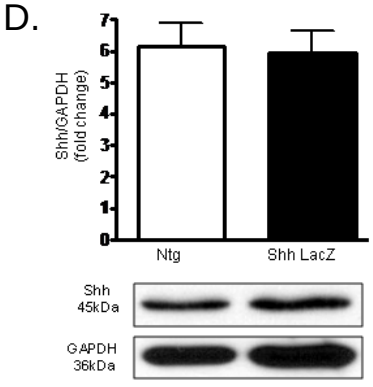
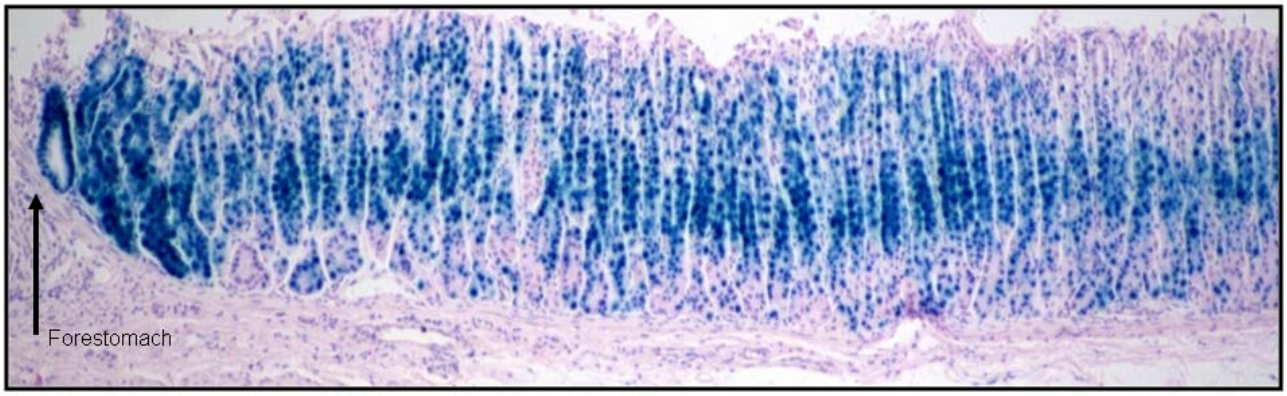

D.
Shh/GAPDH (fold change)
7
6
5
4
3
2
1
0
Ntg
Shh LacZ
Shh 45kDa
GAPDH 36kDa

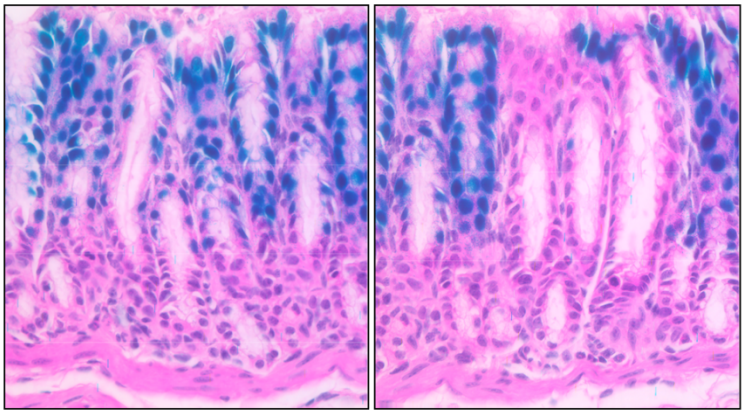
Figure 2.2: Shh is exclusively expressed in the epithelium

Gastric tissue sections extending from the proximal portion of the stomach, the forestomach (black arrow) into the corpus (A) and distal portion of the stomach, antrum (B) are shown. Nuclear β-galactosidase activity was detected in the epithelial cells (X-gal and haematoxylin-eosin staining). RT-PCR analysis of known epithelial (HK-ATPase and gastrin) and mesenchymal (Actg2) markers demonstrate the enrichment of each fraction. Shh mRNA is exclusively epithelial in both corpus (C, 1, 2) and antrum (D, 1, 2). Red rectangle indicates clean separation

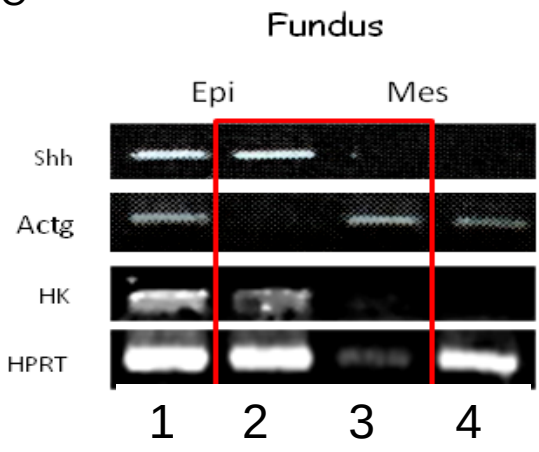
A.


Forestomach

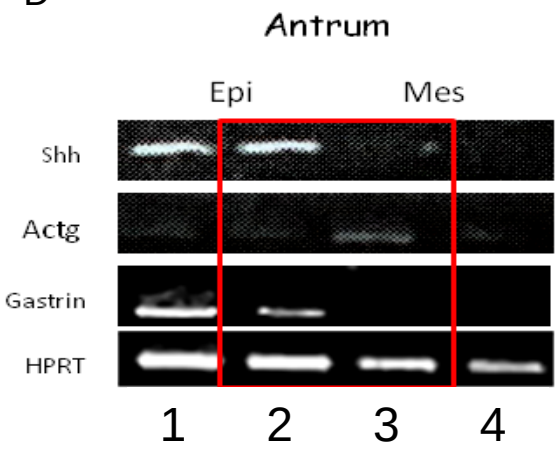
B.



C


Fundus
Epi
Mes
Shh
Actg
HK
HPRT
1
2
3
4

D


Antrum
Epi
Mes
Shh
Actg
Gastrin
HPRT
1
2
3
4

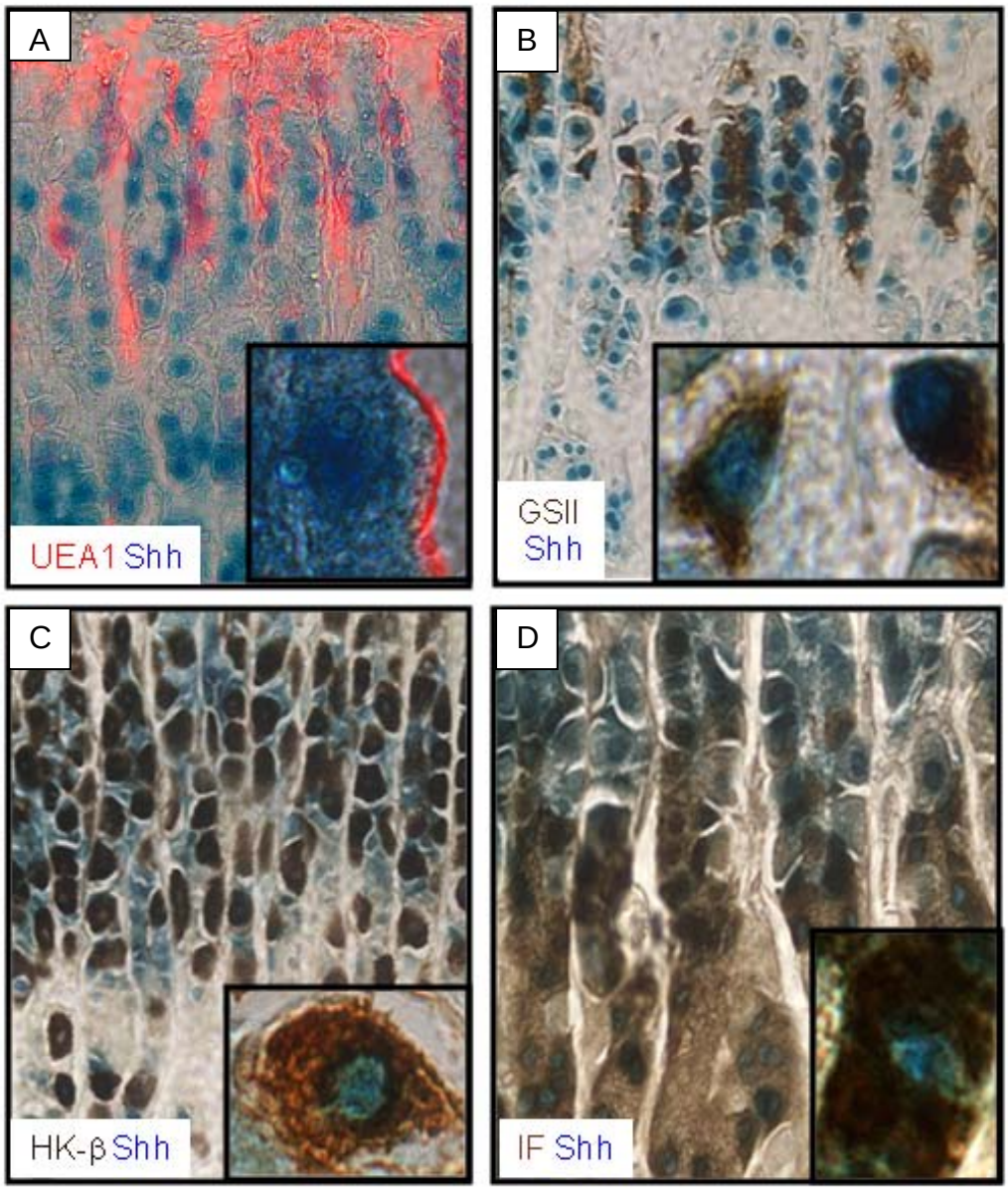
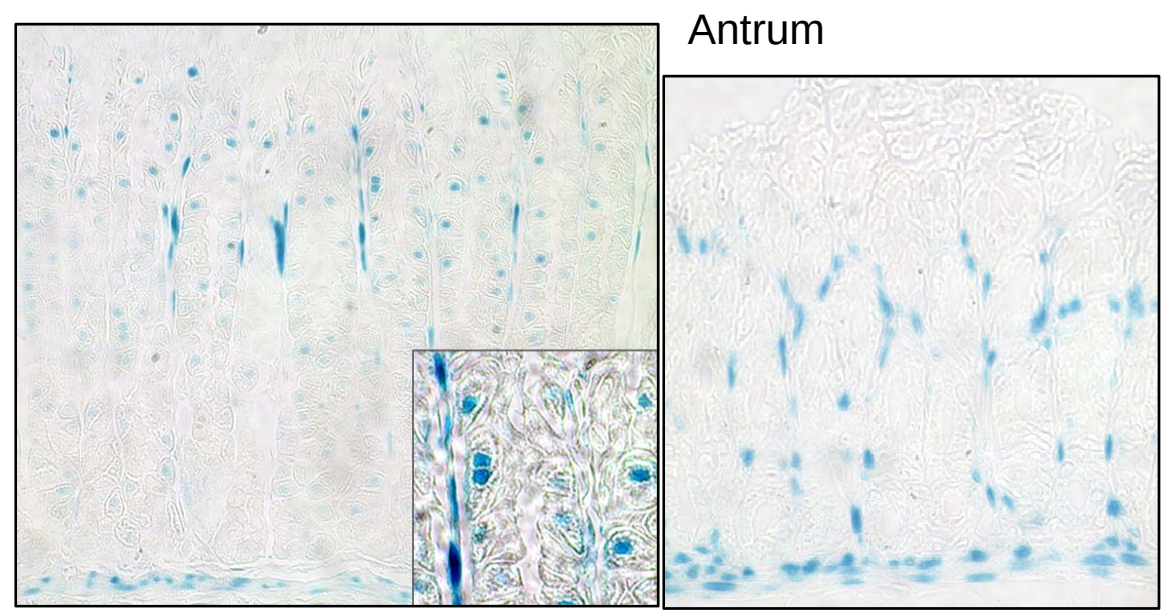


Figure 2.3: Both mucous and oxyntic cell lineages express Shh

Whole mounts of stomachs from Shh-LacZ reporter mice were incubated in the β-galactosidase substrate (X-gal) for 16h prior to paraffin embedding. The stomach was sectioned then immunostained for cell specific markers. Ulex europaeus (UAE1) for pit cells (A), Griffonia simplicifolia (GSII) for mucous neck cells (B), H^+-K^+-ATPase (HK) for parietal cells (C), and intrinsic factor (IF) for chief cells (D). Magnification is 400x; insets are1000x.



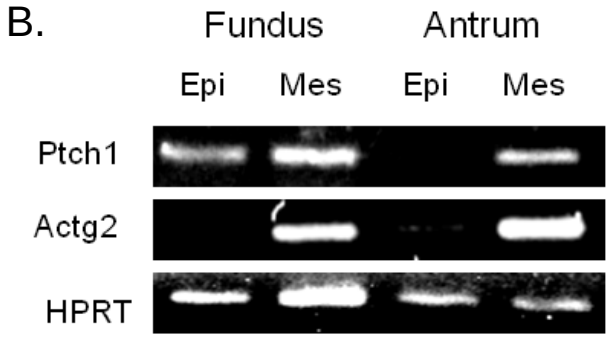


Figure 2.4: Epithelial expression of Ptch1 in the corpus

X-gal staining of Ptch1$^{lacZ/+}$ mice (A). Ptch1 is expressed in the mesenchyme of both corpus and antrum. In addition, corpus epithelium is Ptch1 positive. (B) RT-PCR analysis for known mesenchymal (Actg2) marker demonstrates enrichment of the fraction. Ptch1 mRNA is expressed both in epithelium and mesenchyme in corpus and exclusive in the mesenchyme in the antrum.

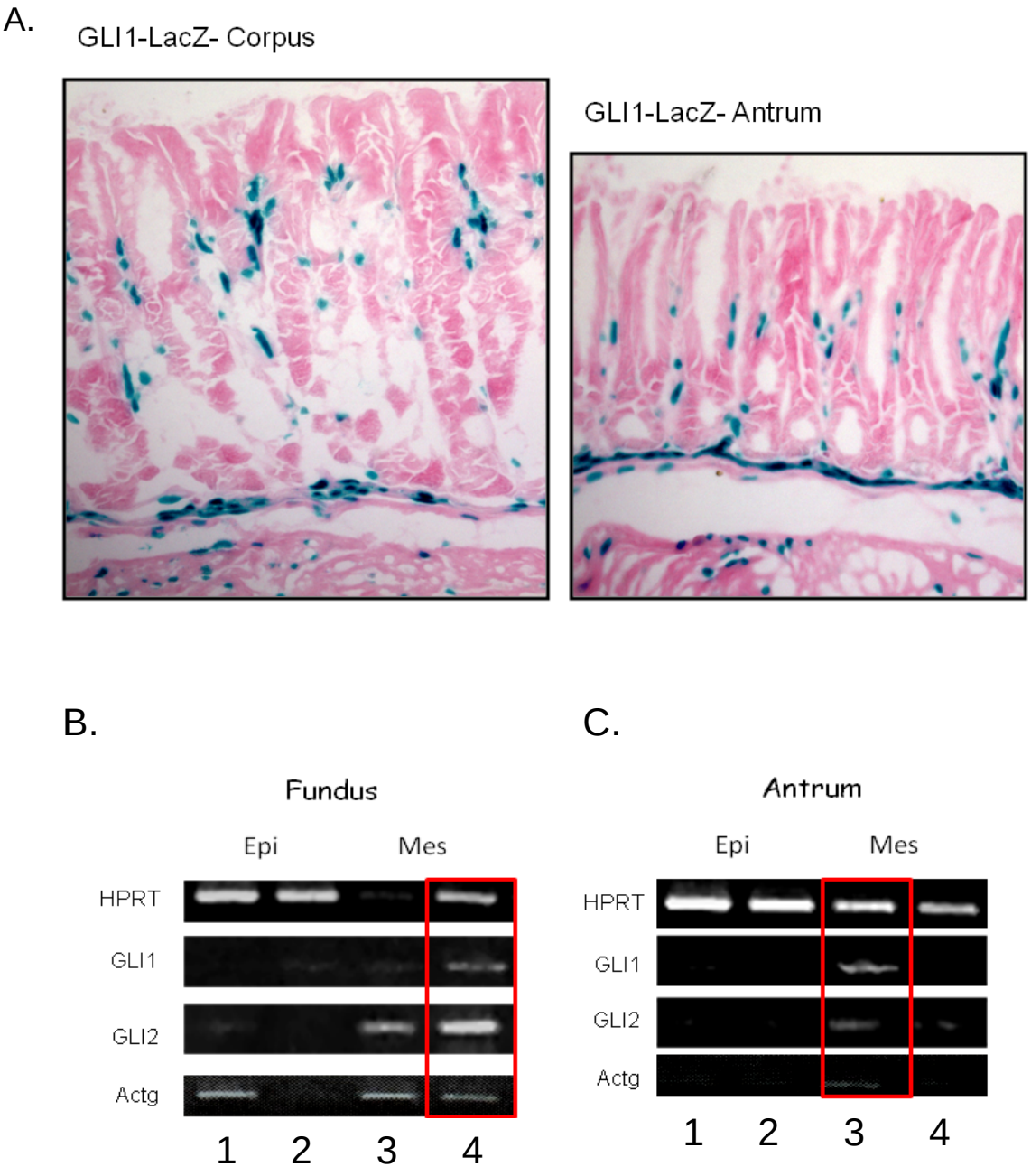


Figure 2.5: Gli1 is exclusively expressed in the mesenchyme

X-gal staining of $Gli1^{lacZ/+}$ mice (A). Nuclear β-galactosidase activity was detected in the mesenchymal cells (X-gal and haematoxylin-eosin staining). RT-PCR analysis of known mesenchymal (Actg2) marker demonstrates enrichment of the fraction. Gli1 and Gli2 mRNA is exclusively mesenchymal in both corpus (B, 3-4) and antrum (C, 3-4). Red rectangle indicates clean separation.

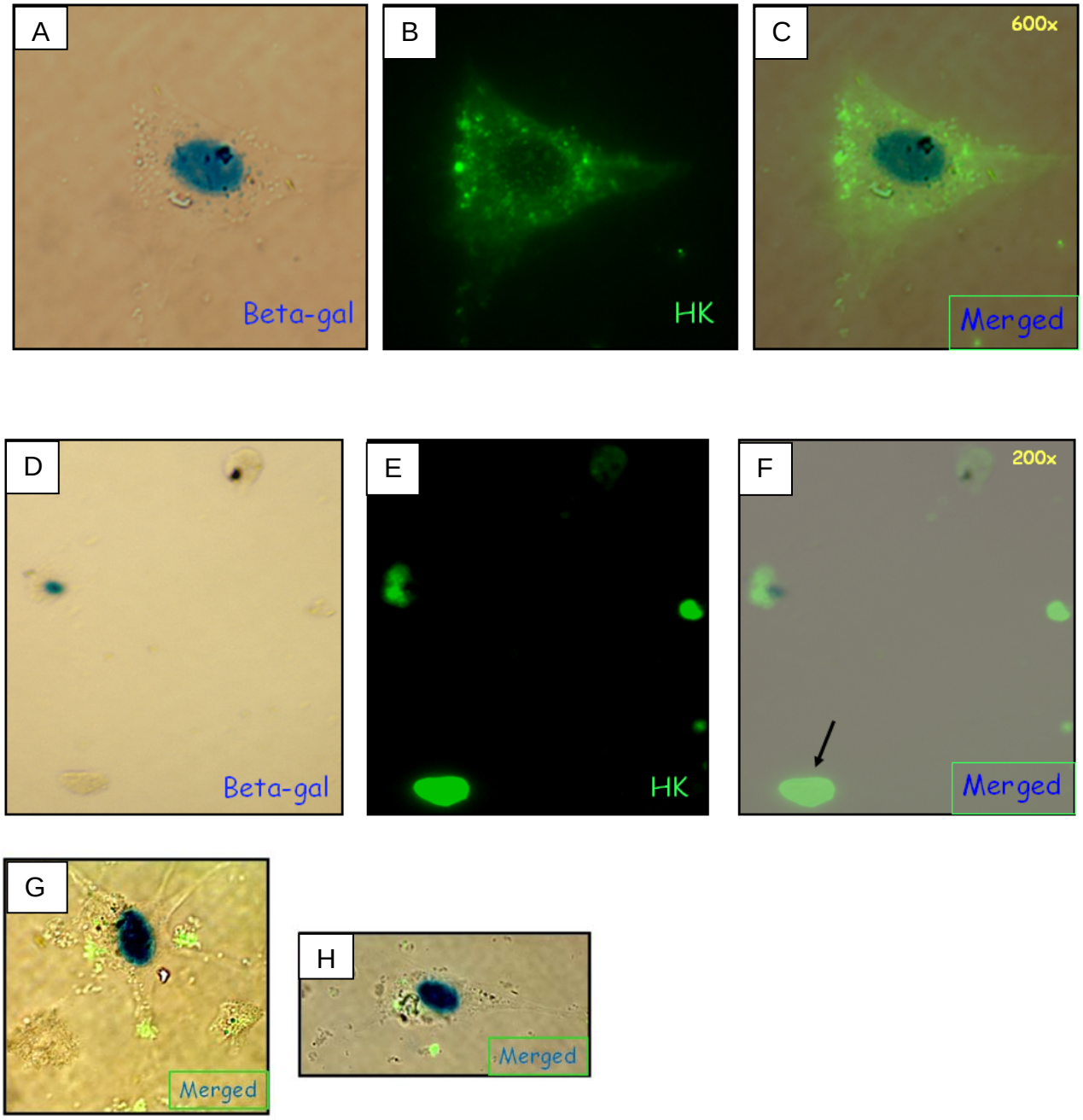


Figure 2.6: Parietal cells express Gli1 *in-vitro*

X-gal and HK-ATPase staining of cells isolated from Gli1$^{lacZ/+}$ mouse stomach cultured and treated with recombinant Shh (1μg/ml) for 24h. Nuclear β-galactosidase activity represents Gli1 expression (A) (D). Parietal cells marked by HK-ATPase expression are shown in green (B) (E). Merged images are shown (C) (F). Arrow represents HK-ATPase positive and β-gal negative cell (F). (G) and (H) are merged images of untreated cells.

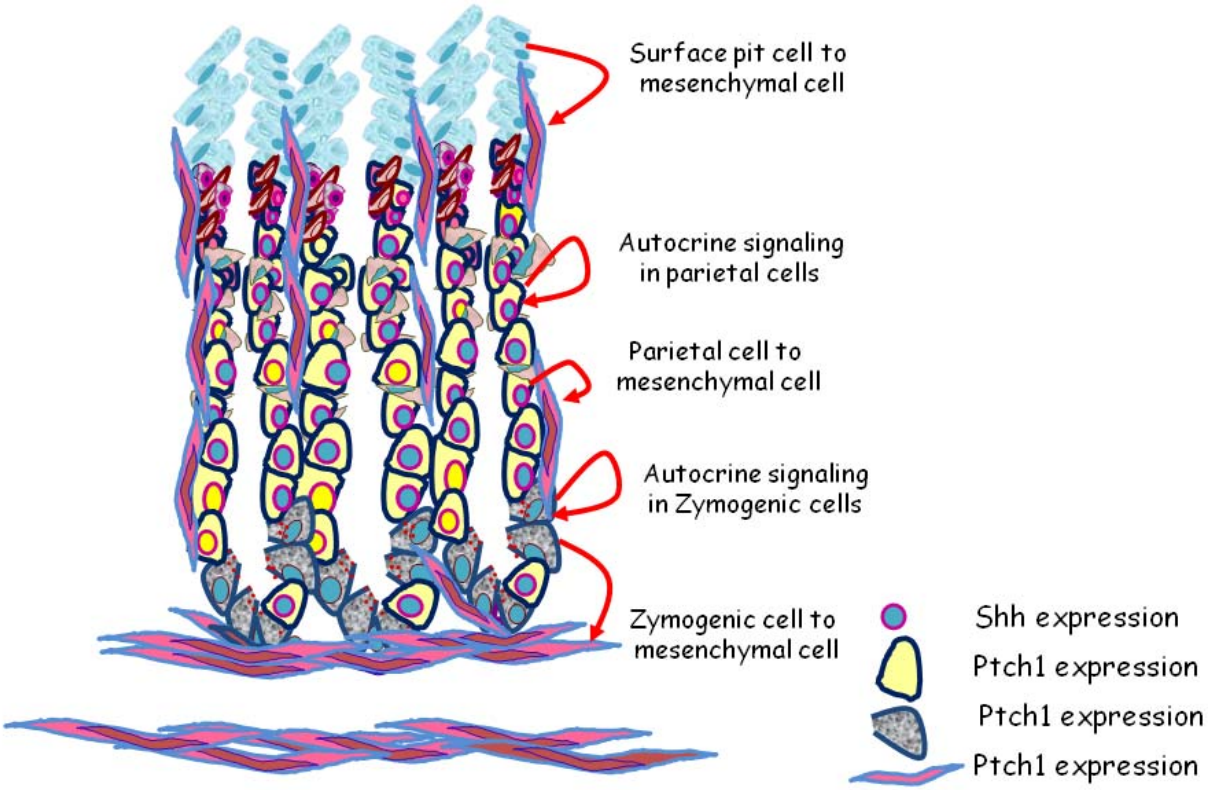


Figure 2.7A: Shh signaling in the adult mouse stomach based on current understanding

Schematic showing the expression of Shh and its signaling components in the adult mouse stomach. The current understanding is solely based on Ptch1 expression and shows that both autocrine and paracrine signaling can occur in the stomach.

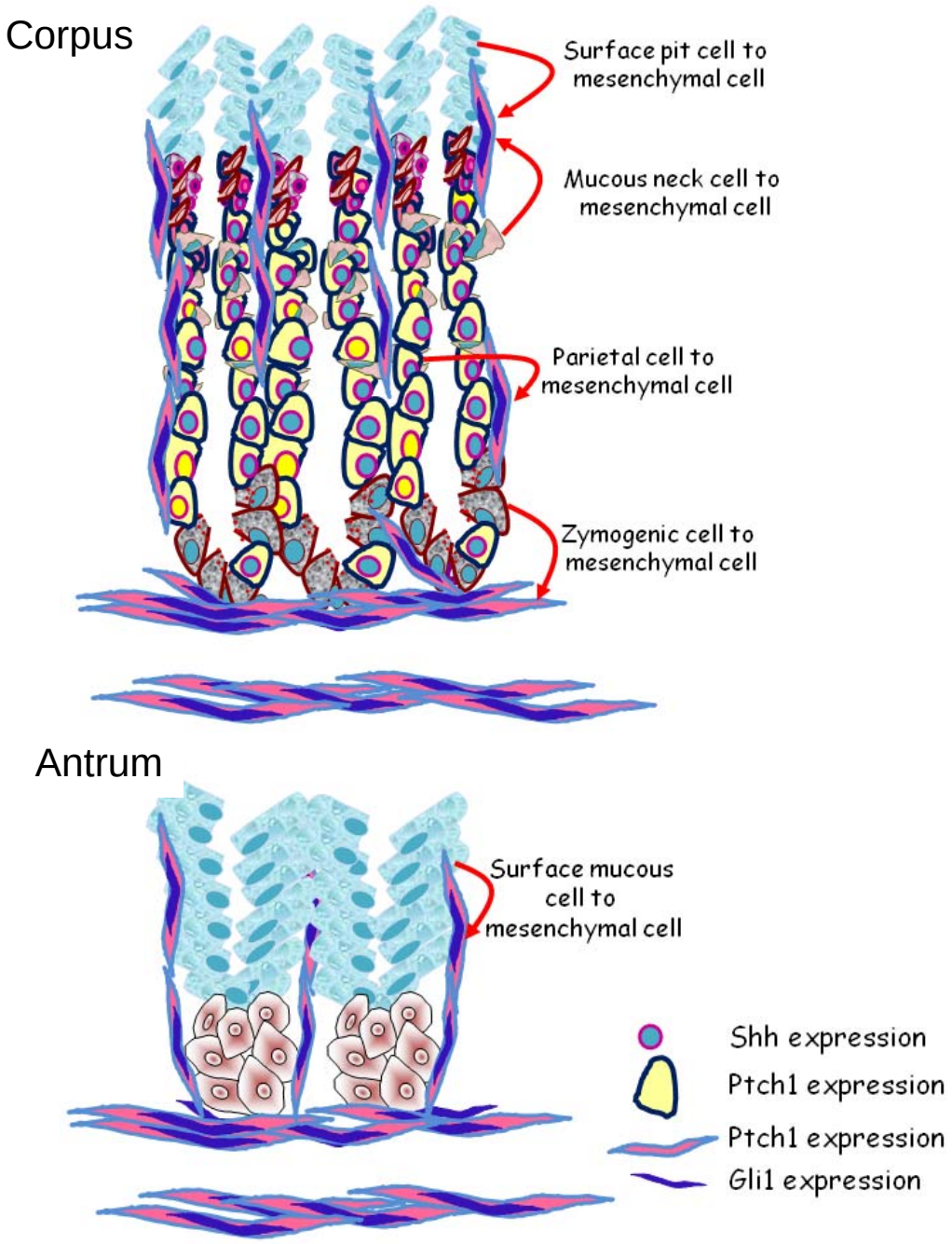


Figure 2.7B: Shh signaling in the adult mouse stomach based on our data

Schematic showing the expression of Shh and its signaling components in the adult mouse stomach corpus (A) and antrum (B). We show expression of Shh in the antrum (B) and in addition to Ptch1 we examined Gli1 expression and show that Hh signaling is paracrine in the stomach.

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CHAPTER III

IL-1β PROMOTES GASTRIC ATROPHY BY SUPPRESSING SONIC HEDGEHOG

ABSTRACT

Background & Aims: Several studies confirm that, *Helicobacter* infection leads to a decrease in Shh expression in human subjects and rodent models. However, to date role for specific pro-inflammatory cytokines in the regulation of Shh expression has not been examined. Polymorphisms in the pro-inflammatory cytokine IL-1β, result in higher levels of the cytokine in human subjects with *H.pylori* infection and gastric atrophy. Since *Helicobacter* infection suppresses Shh, we examined if IL-1β also inhibits Shh expression. Methods: Human tissue sections from uninfected and *Helicobacter* infected patients were immunostained for Shh expression. Shh-LacZ reporter mice were infected with *H.felis* for 3 and 8 weeks. Changes in Shh expression were monitored using β-galactosidase staining and immunohistochemistry. Gastric acidity and IL-1β levels were measured after infection by base titration and QRT-PCR respectively. Primary cultures of canine parietal or mucous cells were treated with IL-1β. Shh protein was determined by immunoblot analysis. Organ cultures of gastric glands from wild type or IL-1R1KO mice were treated with IL-1β then Shh expression was measured using QRT-PCR. Mice were injected with either IL-1β or omeprazole

prior to measuring Shh mRNA expression and acid secretion. Results: *Helicobacter* infection inhibits Shh expression in parietal cells. Activation of the IL-1 receptor was required to inhibit Shh expression by IL-1β since Shh expression was not affected by IL-1β treatment of IL-1R1KO mice. As previously reported, we found that IL-1β inhibited acid secretion. In addition, we found that IL-1β also inhibited Shh expression in the parietal cells. Proton pump inhibitor omeprazole was a more potent inhibitor of both acid secretion and Shh expression. Treatment with both omeprazole and IL-1β resulted in greater inhibition of Shh expression compared to either treatment alone suggesting IL-1β acts via both acid and independent of acid. Conclusion: The proinflammatory cytokine IL-1β suppresses parietal cell Shh gene expression by both acid dependent and independent mechanisms.

INTRODUCTION

Helicobacter induced gastritis of the corpus leads to hypochlorhydria, and oxyntic gland atrophy, a lesion that predisposes the stomach to cancer (El-Omar, Oien et al. 1997; Uemura, Okamoto et al. 2001). However, the mechanism by which chronic inflammation triggers loss of the parietal cells is not understood.

Atrophy is marked by loss of normal gastric glands and is accompanied by replacement with either gastric (pseudopyloric/ spasmolytic peptide-expressing) or intestinal glands (Schmidt, Lee et al. 1999; El-Zimaity, Ota et al. 2002; El-Zimaity 2006; Tsukamoto, Mizoshita et al. 2006). Thus, there is a shift in cellular composition of the stomach from oxyntopeptic lineages to mucous lineages.

Disturbances in gastric differentiation might play a role in the evolution of gastric atrophy. Sonic hedgehog (Shh) has recently been implicated as a crucial factor in gastric organogenesis and glandular differentiation (van den Brink, Hardwick et al. 2001; van den Brink, Hardwick et al. 2002). Earlier studies of the Shh null mice suggested that intestinal metaplasia (IM) develops in the stomach in the absence of Shh (Ramalho-Santos, Melton et al. 2000). However a more recent study suggested that the stomach exhibits features of hyperplasia in Shh null mice rather than IM since the stomach exhibited gastric markers (Kim, Huang et al. 2005). Nevertheless, both studies demonstrate that the gastric mucosa is abnormal in the absence of Shh.

Polymorphisms in the promoter of the pro-inflammatory cytokine IL-1β that increase gene expression correlate with gastric atrophy and cancer in

Helicobacter-infected subjects (El-Omar, Carrington et al. 2000). Recently, gastric transgenic overexpression of human IL-1β was shown to induce dysplasia and gastric cancer (Tu, Bhagat et al. 2008). Apart from its role in inflammation, IL-1β inhibits gastric acid secretion (El-Omar 2001) and hence in this way might hasten atrophy. Prior studies have suggested a role for gastric acid in regulating Shh expression (Dimmler, Brabletz et al. 2003; Minegishi, Suzuki et al. 2007; Zavros, Waghray et al. 2007; El-Zaatari, Grabowska et al. 2008). Therefore, we determined if IL-1β induces gastric atrophy -by suppressing Shh expression.

MATERIALS AND METHODS

Mice

Shh LacZ mice have been described elsewhere (Jeong, Mao et al. 2004). This line carries the beta galactosidase cDNA inserted into the 3' UTR of the Shh locus. The mice were maintained as a homozygous colony. All mice were fasted overnight with free access to water before analysis. The study was performed with the approval of University of Michigan Animal Care and Use Committee, which maintains an American Association for Assessment and Accreditation of Laboratory Animal Care facility.

X-gal staining

The stomach was opened along the greater curvature and the gastric contents were washed in ice cold PBS. The stomach was fixed in fresh 4% paraformaldehyde/PBS (pH 7.0-7.5) for 1h at 4^{o}C. The stomach was washed three times for 30 min each with β-gal rinse buffer (100mM sodium phosphate pH 7.3, 2mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40) at room temperature. The tissue was incubated for 16h at 37^{o}C in β-gal staining solution (β-gal rinse buffer, 25 mg/ml X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide). The tissue was rinsed with β-gal rinse buffer for 30 min at room temperature, and post-fixed overnight in 10% paraformaldehyde/PBS. The tissue was processed and paraffin-embedded prior to sectioning.

mRNA Analysis

Resected tissue was collected in Trizol reagent (Invitrogen) total RNA was extracted, purified, and DNase treated using the RNeasy kit (Qiagen). Using the iScript cDNA synthesis kit (BioRad), cDNA was synthesized from 1μg of total RNA. QRT-PCR was performed using a BioRad I cycler with SYBR Green dye (Molecular Probes). Each 20 μl reaction contained 2μl of reverse-transcribed product, 1x PCR buffer, $MgCl_2$, 100nM of each primer, 1x SYBR Green, 10nM fluorescein, 200mM dNTPs, and 0.025U of Platinum Taq polymerase (Invitrogen). Each PCR amplification was performed in triplicate wells with the following conditions: 3 min at 95^{o}C, 40 cycles of 9s at 95^{o}C and 1min at 60^{o}C, followed by 1min at 55^{o}C. Melt curve analysis was used to assess the purity of the product. Beacon software (BioRad) was used to design primer sequences, Shh reverse ATCGTTCGGAGTTTCTTGTGAT, Shh forward ATGTTTTCTGGTGATCCTTGCT, GAPDH reverse TAT TAT GGG GGT CTG GGATGG, GAPDH forward TCA AGA AGG TGG TGA AGC AGG and TaqMan primer (Applied Biosystems) for IL-1β and GAPDH were used. Data for each gene were normalized to the expression of GAPDH.

Immunoblot analysis

Protein was loaded on a 4-20% SDS-PAGE gradient gel for immunoblot analysis. The membranes (Hybond-C Extra Nitrocellulose, Amersham Biosciences) were blocked with Detector Block (KPL, Gaithersburg, MD, 71-83-00) for 1h at room temperature followed by an overnight incubation with a 1:200

dilution of the goat polyclonal anti-Shh (Santa Cruz Biotechonology, sc-1194), or a 1h incubation with1:5000 GAPDH (Chemicon) antibody. The membranes were washed twice for 10 min each in 1x TBST and incubated for 1h with a 1:5000 dilution of horseradish peroxidase-conjugated secondary anti-goat, or anti-mouse antibodies. The membranes were washed five times for 30 min with 1xTBST. Proteins were visualized using enhanced chemiluminescence (Lumilight substrate, Roche Applied Science, Mannheim, Germany).

Immunohistochemical Staining

X-gal stained tissue sections were deparaffinized and rehydrated. The sections were then washed with 1x PBS, blocked with 20% serum and incubated with a 1: 50 dilution of HRP conjugated anti-GSII , a 1:500 dilution of rabbit anti-Intrinsic factor or a 1:125 dilution of mouse anti-H, K-ATPase –β subunit (Medical & Biological Laboratories Co., LTD) for 1h. Staining was visualized with avidin-biotin complexes by using the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine for the substrate (DAB, DAKO). H, K-ATPase staining was performed using the Ark-labeling kit (Dako). Morphometric analysis was performed on Lac Z positive sections for Shh by counting a total of 200 epithelial cells from each of five different high power fields per section per mouse. The results were expressed as percent Shh positive cells. Morphometric analysis for parietal cells was performed by counting a total of H, K-ATPase positive cells and both H, K-ATPase and LacZ positive cells from 5 oriented glands in random fields for each mouse section. The number of LacZ positive parietal cells was expressed as a percent of the

total number of LacZ positive cells per mouse. Wartin-Starry silver staining was performed to confirm bacterial infection by identifying distinctive spiral organisms.

Immunofluorescence

Immunofluorescence staining was performed on 5 μm paraffin sections. Tissue sections were deparaffinized, rehydrated and blocked with 20% serum. A 1:100 dilution of goat anti-Shh (Santa Cruz), 1:500 dilution of rabbit anti-UEA1, a 1:800 dilution of mouse anti-H,K-ATPase-β subunit (Medical & Biological Laboratories Co., LTD) and anti-IL-1R1 antibodies were used on X-gal stained sections to identify specific cell types followed by a 1:500 dilution of fluorophore–labelled Alexafluor secondary antibody (Molecular probes). Nuclei were counterstained with DAPI and images were generated on a Nikon Eclipse E800 microscope (Nikon, Melville, NY) using a SpotCD camera.

Bacterial Strain and Culture Conditions and Mouse infection

Helicobacter felis (*H. felis*, ATCC, Manassas) was grown on Trypticase Soy Agar with 5% Sheep Blood, plates (BD Diagnostics BBL). Broth cultures were made by inoculating the bacteria in Brucella broth. Mice were gavaged orally inoculated three times over 3 days with 10^8 *H.felis* organisms in 100 μl of brucella broth.

Organ culture

Stomachs from 8 weeks old mice were opened along the greater curvature, and washed in ice cold PBS then twice in ice cold 10% RPMI before

incubating in 6ml of 10% RPMI with either vehicle (PBS) or IL-1β (7.5ng/ml) for 3h.

Primary Canine Cell Preparation and Culture

Canine parietal and mucous cells were isolated based on a modified elutriation method (Soll 1978; Chew, Ljungstrom et al. 1989; Boland, Kraus et al. 1990; Muraoka, Kaise et al. 1996). The isolated parietal cells and mucous cells were cultured in Ham's F-12/Dulbecco's modified Eagle's medium (1:1) containing 0.1mg/ml gentamicin, 50 units/ml penicillin G, 0.01mg/ml ciprofloxacin and 2% DMSO (Sigma) on a 35mm dish coated with 150 μl of growth factor reduced Matrigel (BD Biosciences). The cells were treated with either vehicle (PBS), 100ng IFN-γ or 100ng IL-1β for 6h and whole cell extracts were prepared using RIPA buffer and analyzed by western blot.

Gastric Acidity

The mice were starved overnight. After euthanizing, the stomach was cut along the greater curvature and rinsed with 2ml of normal saline (pH 7.0). Residual gastric debris was removed by centrifugation at 3,000 rpm for 5 min, and the supernatant was collected to determine acidity by titration using 0.005N NaOH. The concentration of gastric acid was expressed as μEq.

IL-1β Treatment

Ten week old wild type mice were injected intraperitoneally with IL-1β (125ng in 100μl/mouse) or vehicle (PBS). The mice were euthanized 3h after receiving IL-1β

Omeprazole Treatment

Ten week old wild type mice were injected intraperitoneally with 10μmol omeprazole**.** The stock solution of omeprazole (80μmol/ml) was dissolved in vehicle dimethyl sulfoxide/polyethylene glycol (4.5/0.5 vol/vol) and stored at -20^{o}C until use. All mice were euthanized 2h after receiving omeprazole or vehicle.

Statistical Analysis

The significance of the results was tested using the unpaired *t* test and one-way ANOVA using commercially available software (Graphpad Prism, GraphPad Software, San Diego, CA). A p value < 0.05 was considered significant.

RESULTS

Helicobacter infection leads to rapid loss of Shh expression

Although *Helicobacter* infection in both human subjects and rodent models leads to decreased Shh expression, role for specific pro-inflammatory cytokines in the regulation of Shh expression has not been examined. Therefore, we infected the Shh-LacZ reporter mice with *H.felis* for three and eight weeks to generate gastritis. Shh expression was reduced at both three weeks and eight weeks of *H.felis* infection (Fig. 1A-D). Bacterial infection was confirmed by Warthin-Starry silver staining of the X-gal stained paraffin sections from the infected mice (Fig. 1E, F). At three weeks of infection, the loss of Shh expression was patchy and correlated with the presence of adjacent areas of inflammation. By eight weeks of infection, the loss of Shh was uniformly distributed throughout the mucosa. Reduced Shh expression was more prominent at the base of the oxyntic glands with surface pit cells still retaining a substantial amount of Shh expression (Fig. 1D). The changes in Shh expression between uninfected and infected mice were quantified using morphometric analysis (Fig. 1G, H). Moreover, the loss of the LacZ staining and therefore Shh expression preceded physical loss of the parietal cells (Fig. 1B, D).

Loss of Shh expression precedes parietal cell atrophy

To confirm that loss of Shh expression preceded parietal cell atrophy, we costained sections from the Shh-LacZ mice with antibodies to the parietal cell marker HK-ATPase and quantified the number of costaining cells by

morphometry. Indeed, there was a decrease in the number of parietal cells that were LacZ positive in response to *Helicobacter* infection (Fig. 2A-C).

To further determine if a similar process occurs in human, we examined the expression of Shh in tissue sections from *Helicobacter*-infected subjects with chronic atrophic gastritis. Shh was strongly expressed in the parietal cells in non-atrophied mucosa of *H.pylori* infected patients with gastritis (Fig. 2D). In subjects with atrophic gastritis, Shh expression was retained where there was normal glandular structure. Similar to the LacZ reporter mice infected with *H.felis* (Fig. 1D), Shh protein expression in parietal cells was reduced, with foveolar epithelial cells still showing substantial amount of Shh expression (Fig. 2E).

IL-1β inhibits Shh expression in parietal cells

Since *Helicobacter* infection suppressed Shh expression in the parietal cells, we examined if IL-1β also inhibited its expression. Time course analysis of IL-1β mRNA expression after three and eight weeks of *Helicobacter* infection was performed. There was a 3 and 5 fold increase in IL-1β mRNA expression respectively (Fig. 3A, B). To demonstrate that the elevated IL-1β levels observed with *Helicobacter* infection was able to suppress Shh expression, organ cultures from the stomachs of wild type and IL-1R1KO mice were treated withIL-1β for 3h. IL-1β suppressed Shh mRNA expression in wild type mice. The inhibition of Shh expression by IL-1β was prevented when cultures were prepared from IL-1R1KO mice (Fig. 3C). Therefore, -I concluded that IL-1β, like *Helicobacter* infection inhibits Shh gene expression.

To identify the cells capable of binding and responding to IL-1β, tissue sections from wild type mice were co-stained with anti-IL-1R1 antibody and the parietal cell marker H, K-ATPase. Both mucous and oxyntic cell lineages expressed the IL-1R1receptor (Fig. 4A, B). To determine which cell type was responding to IL-1β, -I prepared both primary canine parietal and mucous cells. Primary canine parietal cells were cultured and treated with IFNγ, and IL-1β for 6h and protein was prepared for Western blot. Although IFNγ significantly induced Shh expression, IL-1β treatment drastically inhibited Shh expression in parietal cells (Fig. 4C). By contrast, IL-1β treatment had no effect on primary mucous cell cultures (Fig. 4D).

IL-1β inhibits Shh expression by both acid dependent and independent mechanism

It is well established that IL-1β inhibits parietal cell acid secretion (El-Omar 2001). Moreover, deletion of the H, K-ATPase α or β subunit gene locus results in profound hypochlrohydria, atrophy and alters gastric epithelial cell differentiation (Franic, Judd et al. 2001; Judd, Andringa et al. 2005). Prior studies have suggested a role for gastric acid in regulating the expression of Shh (Dimmler, Brabletz et al. 2003). Therefore, we examined if parietal cell specific inhibition of Shh by IL-1β is mediated by the inhibition of acid secretion. Both *Helicobacter* infection and IL-1β reduced acid production (Fig. 5A, B). To assess if IL-1β alone mimicked the effect of *Helicobacter* infection on Shh expression, mice were injected with IL-1β. We found that IL-1β also inhibited Shh expression

(Fig. 5B, C) To investigate if inhibition of acid secretion was sufficient to inhibit the degree of Shh expression seen with IL-1β, I treated the mice with the proton pump inhibitor omeprazole (Fig. 5B). Similarly, omeprazole replicated the effects of IL-1β by inhibiting the expression of Shh (Fig. 5B, C). If IL-1β suppressed Shh by both acid-dependent and independent mechanisms, then -I predicted that both omeprazole and IL-1β treatment would inhibit Shh expression to a greater extent than either agent alone. Indeed, there was an additive repressive effect on acid and Shh expression when the mice received both IL-1β and omeprazole (Fig. 5B, C). This result supported the notion that IL-1β suppresses Shh expression through both its ability to inhibit acid secretion and through acid-independent mechanisms.

DISCUSSION

Helicobacter infection in humans usually occurs in childhood and the infected individuals remain asymptomatic. Most of the *Helicobacter*- related diseases are seen in adults emphasizing the need for the development of prolonged persistent infection and chronic inflammation. Usually the location of colonization by the bacteria determines the outcome of the disease. Antral colonization leads to an increase in gastrin secretion and hence hyperacidity, leading to duodenal ulcers. By contrast, colonization of the corpus results in loss of parietal cell function and atrophy. The mechanism of how chronic inflammation in the corpus from *Helicobacter* infection leads to atrophy is not well determined.

Recently, Shh has been implicated as a crucial factor for gastric gland organogenesis and gland differentiation. Several studies have shown that both in human subjects and rodent models, *Helicobacter* infection leads to a decrease in Shh expression. However, none of the studies to date have examined a role for specific pro-inflammatory cytokines in the regulation of Shh expression.

Because a list of potential cytokine candidate genes would be extensive, we focused on a gene, IL-1β, that is highly relevant to gastric physiology in terms of parietal cell biology. IL-1β is also upregulated in presence of *Helicobacter* infection and has a profound pro-inflammatory effect (El-Omar 2001). It is one of the earliest and most important pro-inflammatory cytokines in the context of *Helicobacter* infection and is also a powerful acid inhibitor (El-Omar 2001).

We observed a rapid loss in Shh expression in the parietal cells within three weeks of *Helicobacter* infection. The loss of Shh was patchy and correlated with focal areas of inflammation. However, there was still a considerable amount of Shh expression seen in the stomach. Infection of mice for two months resulted in a uniform loss of Shh expression from the parietal cells. The loss of Shh expression did not correlate with the presence of bacteria.

It was not known if the loss of Shh expression during *Helicobacter* infection reported thus far was due to loss of the Shh expressing cells or due to inhibition in Shh expression. To the best of my knowledge, this is the first report demonstrating the loss of Shh expression prior to the loss of parietal cells both in human and mouse model of *Helicobacter* infection. Interestingly, loss of Shh expression in the parietal cells is accompanied by an increase in the expression of Shh in the surface mucous cells in humans. Earlier studies have demonstrated increase in Shh expression in mucous cells with chronic gastritis (Dimmler, Brabletz et al. 2003; Ma, Sun et al. 2006).

Since IL-1β, has an inhibitory effect on parietal cell acid production, I sought to determine if IL-1β also inhibited Shh expression in these cells. Organ cultures were treated with IL-1β to study the effect on Shh mRNA expression. Interestingly, I found that IL-1β inhibited the expression of Shh with three hours of treatment. This effect of IL-1β is not unique to Shh; cytokines are known to exert rapid changes in gene expression (Kim, Kwon et al. 2000). I further demonstrated that the effects on Shh are specific to IL-1β, using IL-1R1 null

mice.

To test if IL-1β directly induces its effects, I used isolated primary canine parietal or mucous cell cultures. First I studied the expression of IL-1R1 receptor in the stomach, and found that cells of both oxyntopeptic and mucous lineages express the receptor for IL-1β. Next, I treated the parietal and mucous cell cultures with IL-1β, and measured the expression of Shh. Intriguingly, the parietal cells showed a dramatic inhibition in the expression of Shh; whereas, the mucous cells did not show any significant difference in the expression of Shh upon stimulation with IL-1β

There has been a sudden interest in studying the regulation of Shh by gastric acid. Several reports now have correlated the expression of Shh with reduced acid levels (Dimmler, Brabletz et al. 2003; Minegishi, Suzuki et al. 2007; Zavros, Waghray et al. 2007; El-Zaatari, Grabowska et al. 2008). IL-1β is a known potent inhibitor of gastric acid secretion. Therefore, I hypothesized that IL-1β might be inhibiting the expression of Shh by inhibiting gastric acid production. I injected the mice with IL-1β to test this hypothesis. I also tested to see if omeprazole, a proton pump inhibitor, could mimic the effects of IL-1β. Therefore I injected IL-1β alone and in combination with omeprazole to determine its effect on Shh expression. Omeprazole was more potent at inhibiting acid compared to IL-1β. Both IL-1β, and omeprazole treatments inhibited Shh expression. Interestingly, I observed an additive effect on inhibition of Shh expression in the mice receiving both IL-1β and omeprazole, suggesting that IL-1β acts both in an

acid-dependent and -independent manner.

This present study has significant clinical implications. I have demonstrated that the combined effect of inflammation and omeprazole is sufficient to completely abolish the expression of Shh. These findings warn against excessive or chronic use of antisecretory agents, such as proton pump inhibitors or H_2 receptor antagonists, especially in the presence of other risk factors, such as inflammation caused by *Helicobacter pylori*.

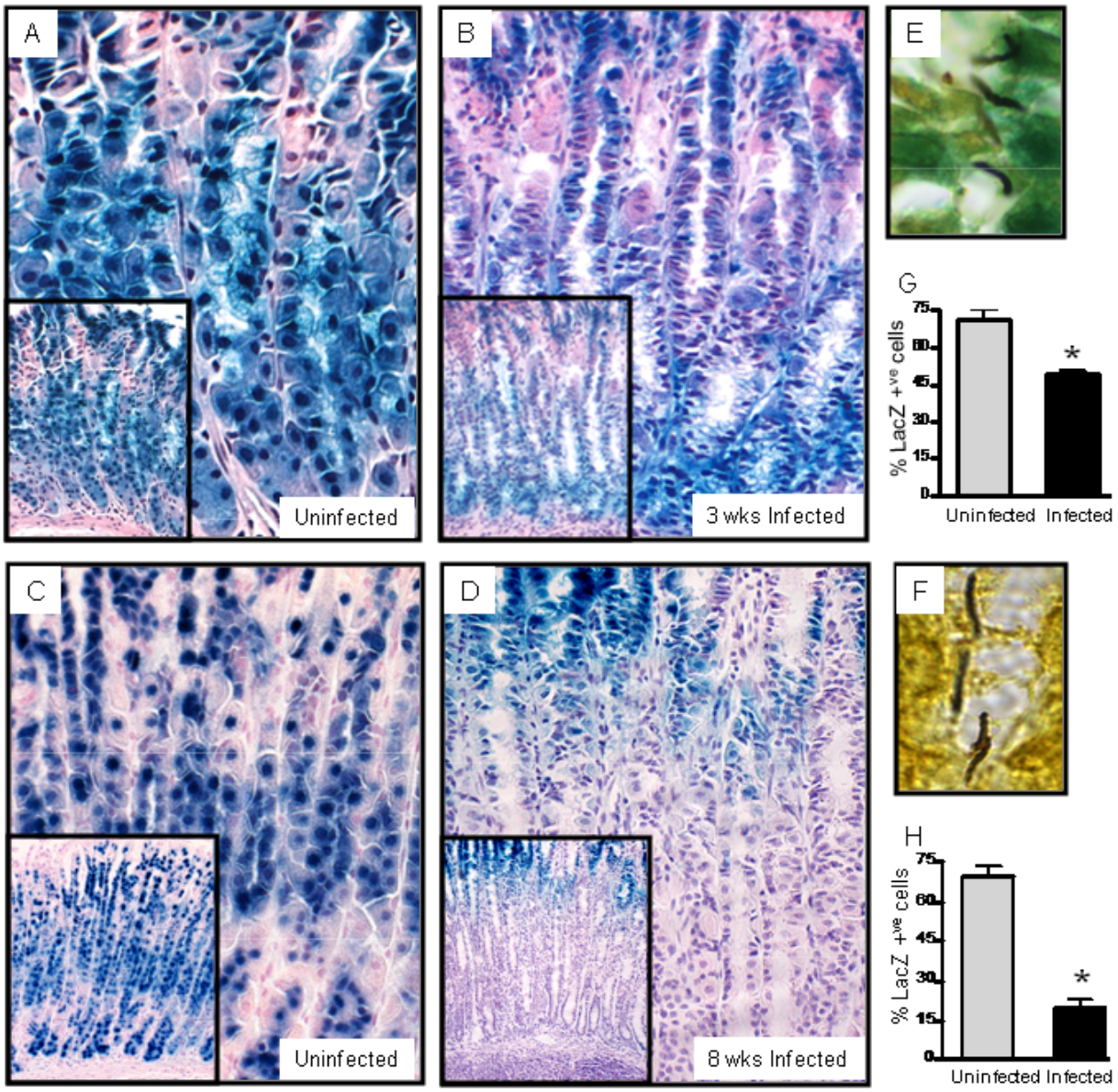
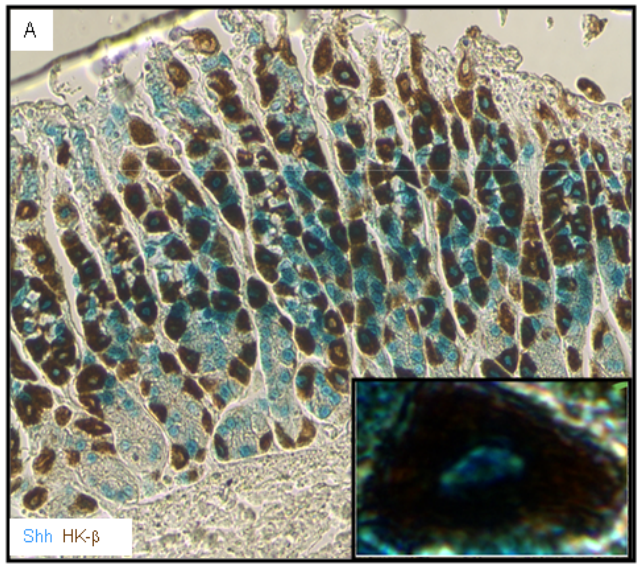


Figure 3.1: Helicobacter infection suppresses Shh expression

X-gal and haematoxylin-eosin staining of three months old Shh LacZ uninfected (A) and mice infected with *H.felis* for three weeks (B) and four months old uninfected (C) and mice infected with *H.felis* for eight weeks (D). Magnification is 200x and the insets are100x. A Warthin-Starry silver staining of X-gal stained paraffin sections from mice infected for three weeks with *H.felis* (E) and eight weeks (F). Magnification is 1000x. Changes in Shh-LacZ positive cells after three weeks (G) and eight weeks of *H.felis* infection were analyzed by morphometry (H). Shown is the mean ± SEM for LacZ positive cells from six mice per group for three weeks and ten mice per group for eight weeks *, $p < 0.05$ relative to uninfected mice.

Figure 3.2: Loss of Shh expression precedes parietal cell atrophy

Immunohistochemical staining of parietal cells on X-gal stained paraffin sections from uninfected and eight weeks *H.felis* infected mice (A). Magnification is 200x, 1000x for insets. (B) Morphometric analysis of total number of parietal cells from uninfected and eight weeks *H.felis* infected mice. Changes in the parietal cells expressing Shh (X-gal) were analyzed between uninfected and eight week infected *H.felis* mice. Shown is the mean ± SEM for fifteen glands from three mice per group *, $p < 0.05$ relative to uninfected mice. Shh expression in non-atrophied mucosa of *H.pylori* infected patients with gastritis (D). The β-subunit of H, K,-ATPase was used to detect the parietal cells (FITC, green), Shh (red) and the nuclei are stained with DAPI (blue). The merged view is shown. Shh expression in the gastric corpus of a different human subject infected with *H. pylori* with atrophic gastritis (E). The merged image view is shown, parietal cells (FITC, green), Shh (Texas red) and the nuclei (DAPI, blue). Parietal cells with reduced Shh expression are indicated with black arrows, and the Shh expression in the surface pit cells are indicated with white arrows.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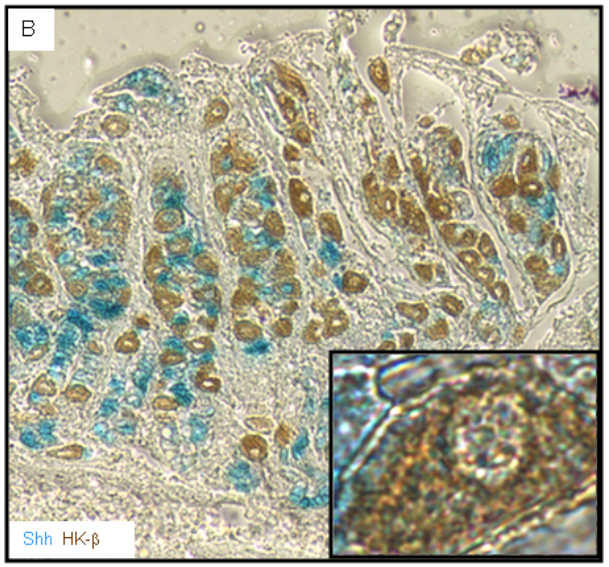
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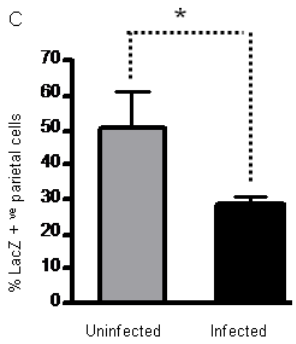
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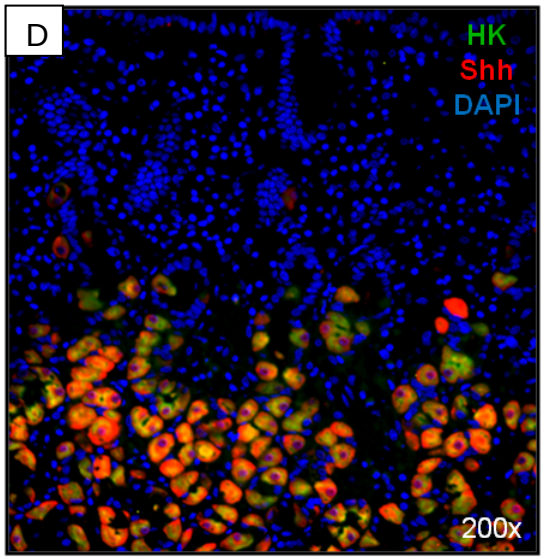
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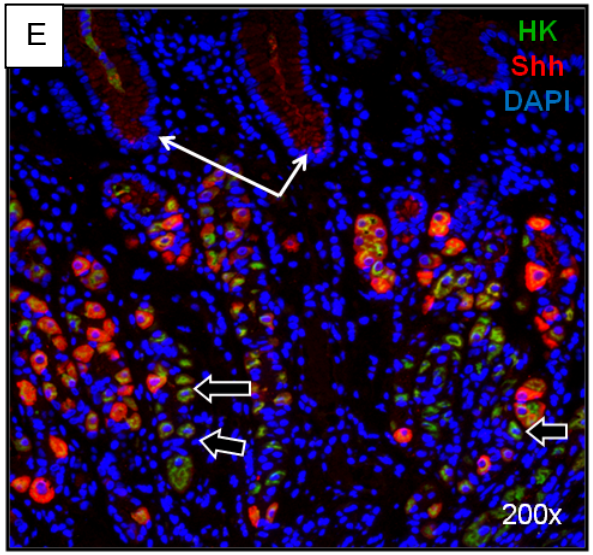
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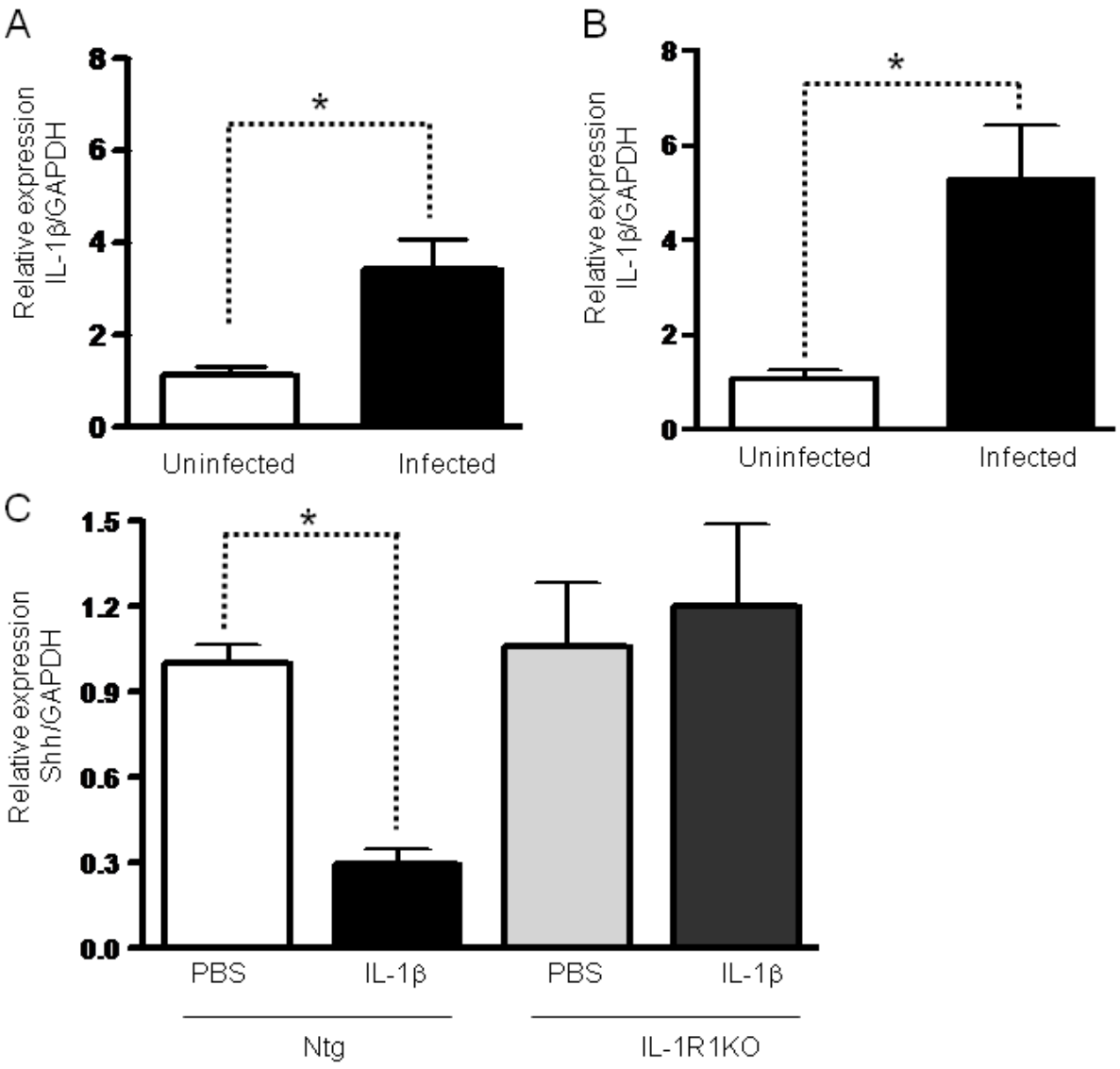

Figure 3.3: IL1R1 is required for inhibition of Shh by IL-1β

Quantitative RT-PCR was performed on total stomach RNA from uninfected and Shh LacZ reporter mice infected with *H.felis* for three weeks (A) (n=6 uninfected, n=4 infected), or eight weeks (B) (n=10). Shown is the relative induction of IL-1β mRNA normalized to GAPDH. The mean ± SEM is shown *, $p< 0.05$ compared to uninfected mice. Quantitative RT-PCR was performed on RNA extracted from organ cultures isolated from gastric corpus of vehicle treated (open bars) or IL-1β treated (filled bars) stomachs isolated from WT or IL1R1KO mice and treated with vehicle (light shaded bars) or IL-1β (dark shaded bars). Shown is the ratio of Shh to GAPDH mRNA expressed as the mean ± SEM for four mice. *, $p< 0.05$ compared with vehicle treated mice.

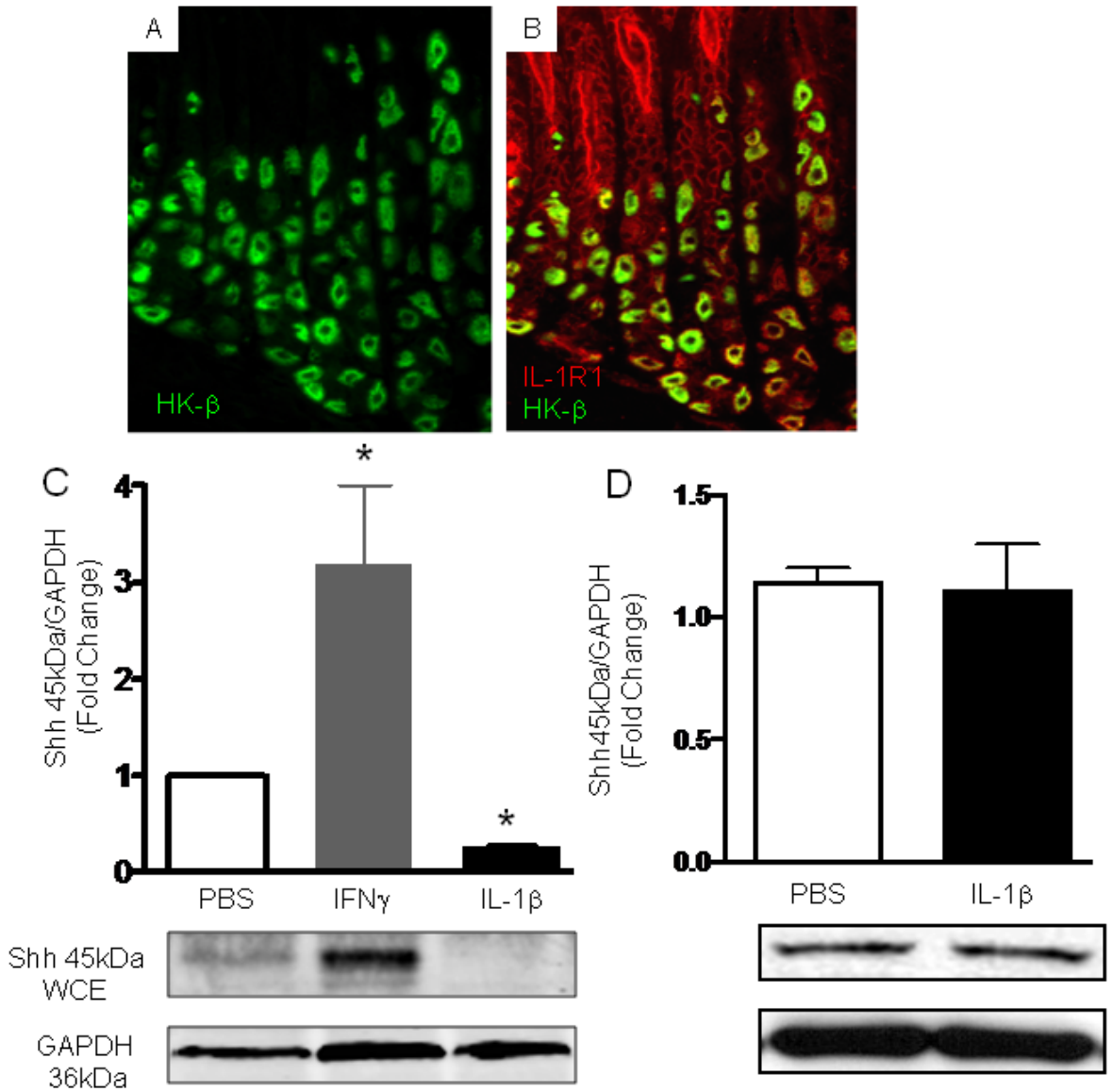

Figure 3.4: IL-1β inhibits Shh expression in parietal cells

Co-localization of H, K-ATPase (green) (A) and IL-1β receptor 1(red) (B). Primary cultures of canine parietal cells were treated with PBS, IFNγ and IL-1β. Whole cell lysates were analyzed by immunoblot (C). Primary cultures of canine mucous cells were treated with PBS and IL-1β. Whole cell lysates were analyzed by immunoblot (D). Shown is the mean ± SEM for three separate parietal cell and mucous cell preparations. *, $p < 0.05$ compared with PBS treatment. ND is not detected.

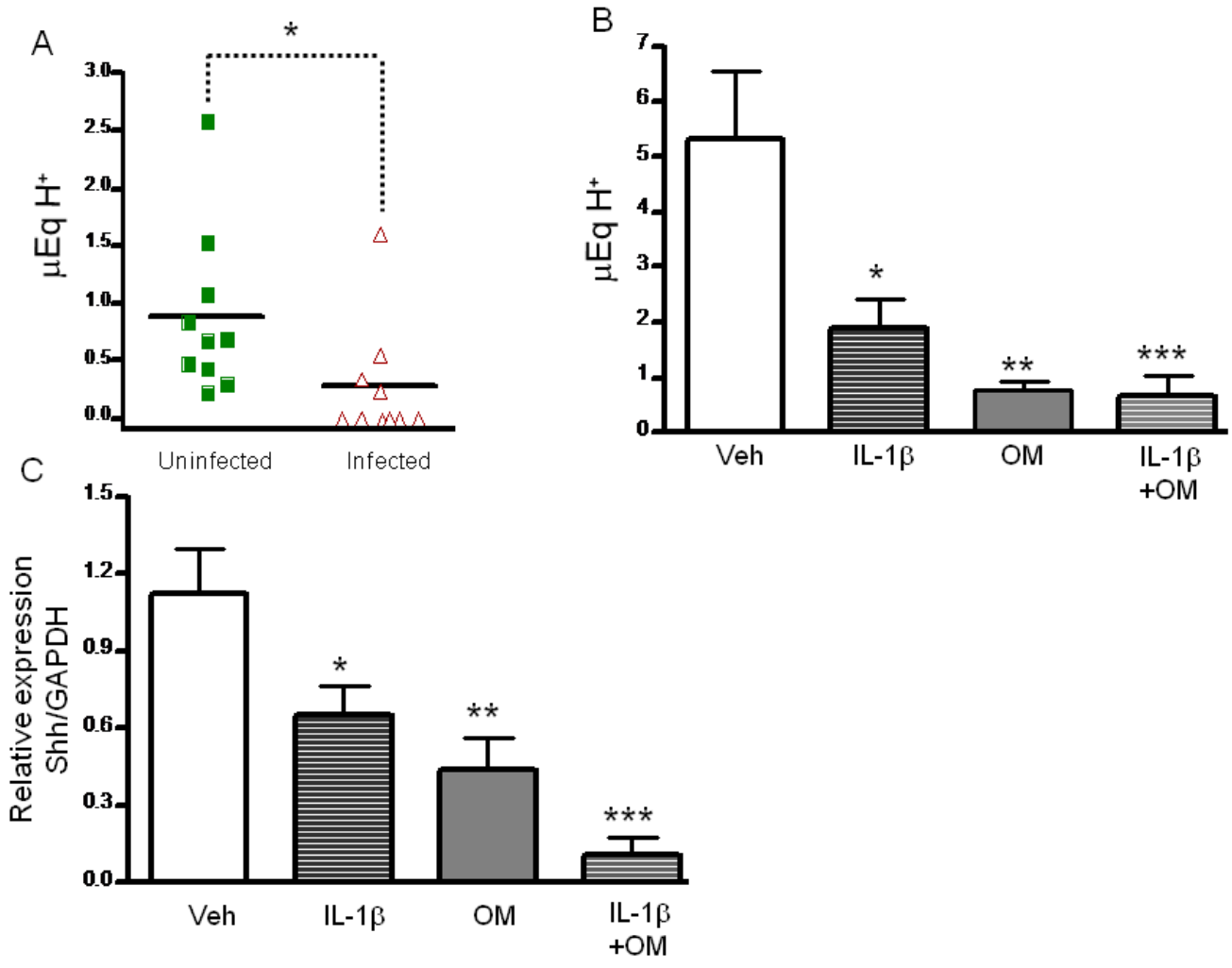

Figure 3.5: IL-1β inhibits Shh expression by both acid dependent and independent mechanism

Gastric acid levels were determined in uninfected and eight week *H.felis* infected mice (A). Acid secretion from mice injected with vehicle (open bar), IL-1β (stripe, filled bar), omeprazole (light shaded) and omeprazole and IL-1β (white stripe filled bar) was measured expressed as µEq of acid H^+ (B). Shown is the mean ± SEM. *, $p < 0.05$ relative to uninfected or vehicle treated mice. Quantitative RT-PCR was performed on total corpus RNA from vehicle (open), IL-1β (stripe, filled bar), omeprazole (light shaded) and omeprazole and IL-1β (white stripe filled bar) treated mouse stomachs. Shown is the ratio of Shh mRNA to GAPDH mRNA (C). The mean ± SEM for eight mice is shown. *, $p< 0.05$ compared with vehicle treated mice

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CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Infection with *Helicobacter pylori* elicits an aggressive Th1 pro-inflammatory response resulting in gastritis which precedes a series of morphological changes that leads to gastric cancer. Treatment with antibiotics to eradicate *H.pylori* early in the pathological process might prevent the progression of the disease. However, the benefit of *H.pylori* eradication in patients with advanced premalignant lesions showing irreversible mucosal changes is questionable.

Polymorphisms in the pro-inflammatory cytokine IL-1β, which correlate with higher levels of the cytokine predispose *Helicobacter*-infected subjects to gastric atrophy, and eventually gastric cancer (El-Omar, Carrington et al. 2000). Although bacterial virulence factors are important, it appears that it is the host's inflammatory response to infection that determines the disease outcome. Therefore, mediators of chronic inflammation can serve as potentially important therapeutic targets to prevent gastric cancer.

The work in this thesis uncovers a molecular pathway that might mediate the progression from chronic inflammation to gastric atrophy. I have established a previously unknown role for IL-1β in gastric patho-physiology. I have

established parietal cell specific inhibition of Shh expression by theIL-1β. Moreover, I have demonstrated that IL-1β inhibits Shh expression by both acid dependent and independent mechanisms.

Non-canonical autocrine Hh signaling

In the experiments described in Chapter II, Shh, Ptch1 and Gli1 reporter mice were analyzed to define the source of Shh and establish the direction of Hh signaling in the stomach. I have demonstrated that Shh is expressed in the corpus and antrum of the adult mouse stomach. The expression of Shh was strictly epithelial and was found in all major epithelial cell types including surface pit, mucous neck, parietal and chief cells. However not all parietal cells expressed Shh. The expression of Ptch1 was seen in both the epithelial and mesenchymal compartments of the corpus but only in the mesenchymal compartment of the antrum. The Hh target and signaling molecule Gli1 was expressed strictly in mesenchyme of the adult stomach. I concluded that canonical Hh signaling is paracrine in normal adult mouse stomach.

Yet, the expression of Ptch1 and the absence of detectable Gli1 expression by the parietal cells in vivo is puzzling. Recently, Osawa et al. demonstrated that Shh signaling might occur strictly through the Smo/Ptc receptor in rat gastric mucosal cells (Osawa, Ohnishi et al. 2006). Shh was shown to elevate intracellular calcium concentration in a transcription-independent pathway via the patched/smoothened receptor system.

Calcium signaling has been shown to be important for the morphologic rearrangements that are characteristic of parietal cells when transforming from the resting to stimulated state (Li and Mardh 1996; Urushidani and Forte 1997). Both, gastrin and histamine stimulate parietal cell acid secretion by, elevating intracellular calcium. Therefore the possibility exists that Shh binds the Ptch receptor on the parietal cells and augments acid secretion (Fig. 4.1). Moreover, this might potentially be the mechanism for reported potentiation of histamine induced acid secretion by Shh (Stepan, Ramamoorthy et al. 2005).

Given this information the next task at hand will be first, to assess for the ability of parietal cells to increase intracellular calcium in response to Shh. This can be achieved by treating either primary mouse parietal cells or primary canine parietal cells with Shh. Isolation of parietal cells from both of these sources is well established (Muraoka, Kaise et al. 1996; Takeuchi, Pausawasdi et al. 1999; Hinkle, Bane et al. 2003). The changes in intracellular calcium upon stimulation with Shh can be measured using fura-2 dye as described by Del valle et al. (Delvalle, Tsunoda et al. 1992) .

Next, it needs to be determined whether Shh induced alterations in intracellular calcium levels depend on transcriptional regulation. Treatment of cells with the transcription inhibitor actinomycin D, will determine if Shh induces intracellular calcium independent of the canonical Gli-mediated transcription pathway. This can further be confirmed genetically by knocking down Gli expression using siRNA in primary parietal cells or by using parietal cells from Gli knockout mice. Once Shh induced increases in intracellular calcium is

established in the absence of Gli then, parietal cells should be treated with cyclopamine to determine if signaling through the Ptch-Smo receptor complex is required for the Shh effects. Thus, these experiments will test for direct effects of Shh on parietal cells, and address the mechanism of non canonical-autocrine Shh signaling in the parietal cells. Moreover, the reported cyclopamine induced hypergastrinemia in mice could be due to reduced acid secretion resulting from reduced Shh signaling in parietal cells (El-Zaatari, Grabowska et al. 2008).

IL-1β mediated acid dependent and independent effects on Shh expression

In the experiments described in Chapter III, I have shown that the proinflammatory cytokine IL-1β suppresses parietal cell Shh gene expression. However, it remains to be determined if IL-1β can directly affect the expression of Shh independent of acid inhibition.

The inhibition of Shh gene expression upon IL-1β treatment was demonstrated both in vitro and in vivo using three different model systems. First, mouse stomach organ cultures treated with IL-1β showed a decrease in Shh expression. Second, the direct effect of IL-1β on parietal cell Shh expression was established by treating isolated primary canine parietal cells with the cytokine. Third, injection of mice with IL-1β, suppressed Shh expression in vivo.

Effects of IL-1β on inhibition of parietal cell function are well established. IL-1β is known to inhibit gastric acid secretion both in vivo in mice and in vitro, in primary parietal cells cultures from rabbit and dog (Wallace, Cucala et al. 1991;

Beales and Calam 1998; El-Omar, Carrington et al. 2000; El-Omar, Carrington et al. 2001). Studies have documented both, a direct inhibitory action of IL-1β on parietal cell acid secretion (Beales and Calam 1998), as well as an indirect effect by inhibiting the release of histamine from ECL cells (Saperas, Yang et al. 1990). Recent studies have focused attention on understanding the mechanism of IL-1β induced inhibition of acid secretion. Since infection with *Helicobacter pylori* suppresses H, K-ATPase gene expression (Gooz, Hammond et al. 2000); it was presumed that the inhibition was mediated by IL-1β inhibition of gastric acid secretion. Recently, the effect of IL-1β on the H, K- ATPase α-subunit (HK-α) gene expression was investigated using promoter-reporter constructs containing human HK-α 5'-flanking sequence deletions (Saha, Hammond et al. 2007). IL-1β-responsive elements were identified downstream of nucleotide-206 in the HK-α promoter. Interestingly, induction of HK-α subunit gene expression by IL1-β required the ERK1/2 kinas signaling pathway (Saha, Hammond et al. 2007). Although, this study suggests, a positive effect of IL-1β on H, K -ATPase gene expression, it lacks convincing evidence to support the claim. The study does not document the effect of IL-1β on the endogenous HK-α subunit gene. Furthermore, this study was done using AGS gastric cancer cells, which do not produce acid or express HK-α subunit. Therefore, one cannot predict what effect IL-1β has on HK-α subunit gene expression in parietal cells, and subsequently, how IL-1β induces inhibition of gastric acid secretion.

Recent studies have correlated gastric acidity with Shh gene expression. One study documented that increasing the acidity of the culture medium for the gastric cancer cell line 23132 is sufficient to induce Shh mRNA expression (Dimmler, Brabletz et al. 2003). H_2 receptor null mice which express low levels of Shh at baseline expressed higher levels of Shh mRNA when the mice were gavaged with HCL (Minegishi, Suzuki et al. 2007). However, it is still not understood how gastric acidity influence Shh gene expression.

Therefore in the experiments described in Chapter III, I tested the hypothesisIL-1β regulates Shh expression independent of acid secretion this hypothesis requires our understanding of the effects of IL-1β on parietal cell. The combined effect of omeprazole and IL-1β on Shh expression and acid secretion was established in an in vivo experiment. Similar experiments need to be performed on canine primary parietal cells to confirm the direct effects of IL-1β and omeprazole. We already know from the experiments described in Chapter III that IL-1β can directly inhibit Shh expression in primary parietal cells. A similar effect of IL-1β on acid secretion in cultured parietal cells has been previously reported (Beales and Calam 1998).

IL-1β primarily signals through the type I IL-1 receptor (IL-1R1) and I have demonstrated that the IL-1β effects on Shh expression are mediated by its receptor. The IL-1R1 receptor binds IL-1β but requires that the IL-1receptor accessory protein (IL-IRAcP) transduce the signal. IL-1 binding, causes activation of IRAK-1 (IL-1Receptor Associated Kinase-1), IRAK-2 (IL-1Receptor

Associated Kinase-2) and MyD88 (myeloid differentiation primary response gene 88). IRAK-1 activates and recruits TRAF6 (TNF-receptor associated factor 6) to the receptor complex. TRAF6 activates two pathways, one leading to NF-kB activation and another leading to c-jun activation (Medzhitov, Preston-Hurlburt et al. 1998; Kopp, Medzhitov et al. 1999; Wang, Deng et al. 2001) (Fig.4.2).

Experiments using both pharmacological inhibitors of this pathway and genetic knockdown or over-expression should then be employed to determine the mechanism of Shh inhibition by IL-1β in primary canine parietal cells. Once it is determined whether NF-kb or c-jun signaling is required for IL-1β– mediated inhibition of Shh, then the specific DNA element in the Shh promoter mediating this effect could be identified. Using in silico analysis, I have already determined that conserved NF-kB and AP-1 binding sites exists within the Shh promoter within the first 5kB upstream of the transcription start site. The DNA response element(s) need to be mapped to a specific region by transfecting the promoter-reporter construct in canine parietal cells. Then finer mapping of the element could be performed by site-directed mutagenesis of the predicted elements within the context of the 5kB promoter. The deletion analyses will identify the elements that are required for the response while the site-directed mutations will assess whether specific elements are sufficient.

Once the response element sequences are identified, oligonucleotide probes for gel shift (EMSA) analysis should be generated. These experiments will identify the complexes binding to the element. Pretreatment of the cells with IL-1β before preparing nuclear extracts for EMSAs would permit assessment of

whether binding is induced by the cytokine. A time course of the induction would need to be determined, a shorter time course would select for direct IL-1β effects as opposed to indirect effects that can occur due to the release of other cytokines, e.g., IL-8. Finally, to define the NF-KB or AP1 element that mediates Shh gene expression in vivo in canine parietal cells in response to IL-1β, ChIP assays using specific antibodies should be performed. Thus, these experiments might address the mechanism for IL-1β–mediated inhibition of Shh expression

Role of Shh in the stomach

Our current understanding of the specific functional role of Shh in adult gastric physiology is solely based on in-vitro studies. The role of Shh in parietal cell physiology was explored using primary canine parietal cells by Stepan et al. Stimulation of parietal cells with Shh was shown to induce the expression of the H,K-ATPase α-subunit, suggesting Shh might be involved in parietal cell differentiation or maintenance (Stepan, Ramamoorthy et al. 2005). The role of Shh on cell proliferation was demonstrated by ligand-activated growth of gastric cancer cell lines (Berman, Karhadkar et al. 2003). The attempts to address the role of Shh in vivo have been limited by the early lethality of the Shh null mice (Ramalho-Santos, Melton et al. 2000; Kim, Huang et al. 2005). Since the development of the stomach is not completed until the third week of postnatal life (Karam, Li et al. 1997) the studies from embryonic Shh null mice might represent its role in stomach development hence, its role in an adult stomach needs to be determined. Several studies have employed the use of the Hh inhibitor,

cyclopamine, in an attempt to understand the function of Shh in an adult stomach (van den Brink, Hardwick et al. 2001; van den Brink, Hardwick et al. 2002; El-Zaatari, Grabowska et al. 2008). As, the expression of all the three ligands Shh, Ihh, and Dhh has been reported in adult stomach (Fukaya, Isohata et al. 2006) the pathway inhibitor studies suggest a role for Hh signaling rather than Shh specifically. Therefore clearly there is a need for the development of a genetic model to study the role of Shh in-vivo in the context of gastric physiology.

Conclusion

The goal of my dissertation was to understand the mechanisms leading to loss of Shh expression during the development of gastric atrophy. Specifically, I was interested in determining the effects of a specific pro-inflammatory cytokine on the regulation of Shh gene expression. Using genetic reporter mice I first characterized Shh expression and signaling in an adult mouse stomach. My findings have identified IL-1β as an important cytokine in regulating Shh gene expression during *Helicobacter* infection. I further demonstrated that, IL-1β induced inhibition of Shh expression was mediated through both acid-dependent and independent mechanisms.

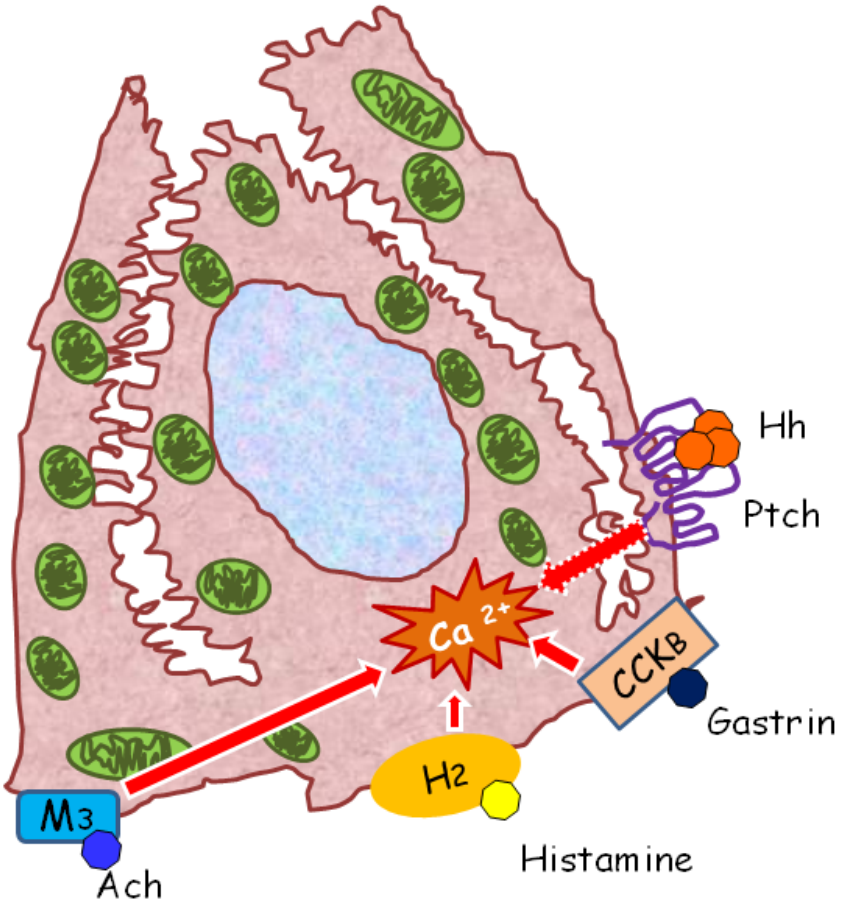

Figure 4.1: Schematic depicting possible non-canonical autocrine Hh signaling in the parietal cell

Parietal cells are stimulated by three main agonist's gastrin, histamine and Ach. Stimulation by these secretogogues increases intracellular Ca^{+2} in the parietal cells. Upon stimulation the H, K-ATPase proteins are exposed to the apical membrane to secret acid. The possibility of Hh signaling to stimulate increase in intracellular Ca^{+2} is shown.

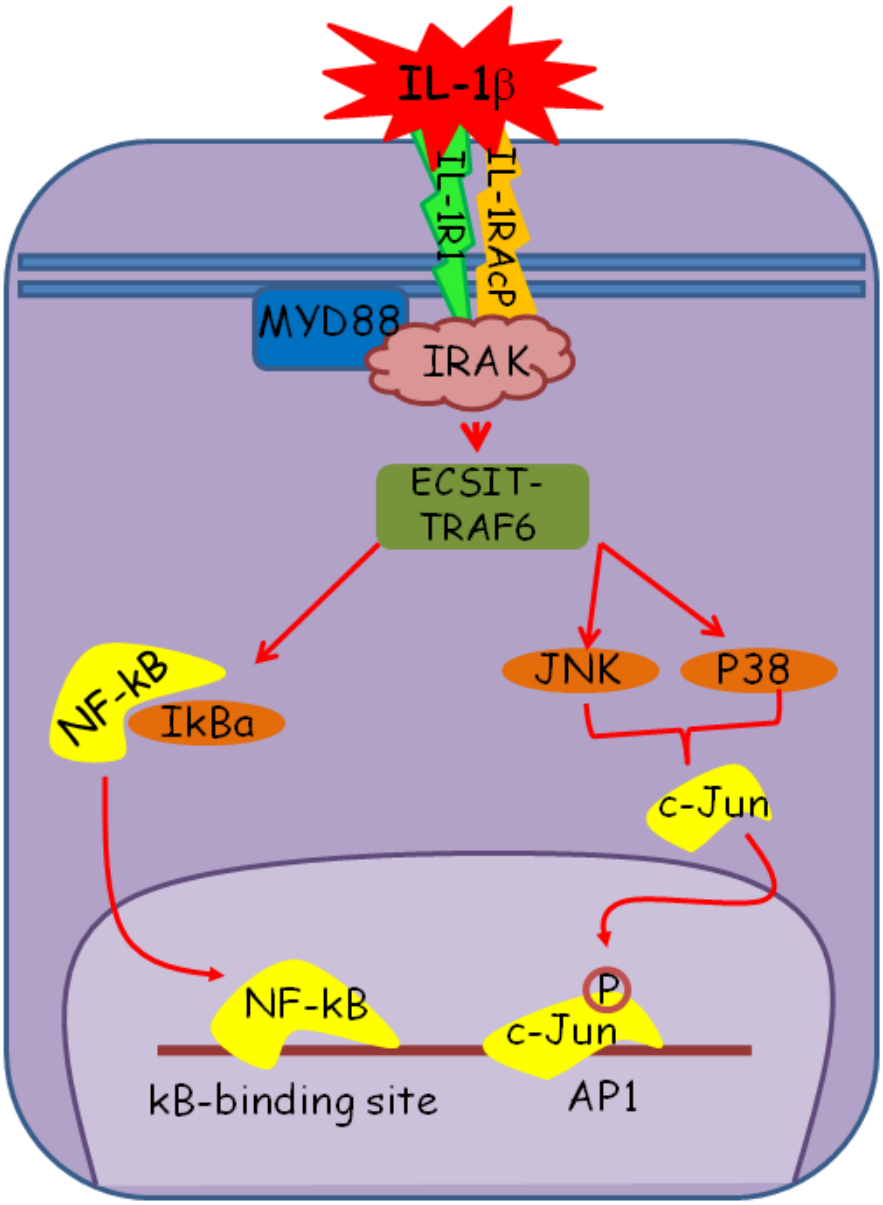

Figure 4.2: IL-1β signal transduction.

Upon IL-1b binding to IL-1R1, IRAK and MyD88 are activated. IRAK-1 activates and recruits TRAF6 to the receptor complex. TRAF6 activates two pathways, one leading to NF-kB activation and another leading to c-jun activation. NF-kB and c-Jun then bind to kB binding element or AP-1 respectively to initiate transcription of target genes.

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APPENDIX

WNT SIGNALING IS ACTIVATED DURING THE PROGRESSION TO GASTRIC ATROPHY AND METAPLASIA

INTRODUCTION

The epithelium of the stomach is organized into gastric units. A sequence of epithelial cell proliferation, differentiation, migration and cell death occur within each gastric unit and is repeated throughout the animal's life span. Stem cells located in the isthmus region give rise to cells that differentiate as they move up or down along the gastric unit. Though the exact molecular steps involved in the proliferation, differentiation and death of these lineages are unknown, an important aspect of this process is the bi-directional crosstalk between the epithelium and the mesenchyme. The Wnt and hedgehog signaling pathways are thought to play a role in this complex morphogenetic process. Upon Wnt binding to the Frizzled receptor, Disheveled and the multiprotein complex comprised of APC, Axin and GSK3-β are recruited to the membrane where Axin associates with LRP5/6 then is subsequently degraded. Disheveled inactivates GSK3-β, leading to stabilization of β-catenin, which translocates to the nucleus. Nuclear β-catenin binds Tcf4, an HMG-type DNA binding protein to activate the transcription of growth-related genes (Huelsken and Behrens 2002). A family of secreted frizzled-related proteins (sFRP) at higher concentrations inhibits Wnt signaling by binding Wnts in the extracellular space disrupting their receptor binding (Ladher, Church et al. 2000; Uren, Reichsman et al. 2000; Yoshino, Rubin et al. 2001). Thus sFRPs primarily function as Wnt inhibitors.

Recently a homeobox domain transcription factor Barx1 was identified using Serial Analysis of Gene Expression (SAGE) (Kim, Buchner et al. 2005). Barx1 is expressed in the stomach mesenchyme during gastric development and has been shown to specify gastric cell identity by inhibiting Wnt signaling (Kim, Buchner et al. 2005). Barx1 null mice show hyperplastic undifferentiated gastric epithelium with the expression of intestine specific genes including Cdx2, Muc2, defensin-2 and FABP. The expression of Wnt antagonist's sFRP1 and sFRP2 was suppressed in these mice. The authors concluded that suppression of the Wnt pathway was essential for appropriate differentiation of the gut tube into normal corpus (Kim, Buchner et al. 2005).

Elevated levels of Shh, its signaling and aberrant accumulation of nuclear β-catenin have been reported in human gastric cancer. However, its functional role has not been established. Recently, hedgehog signaling has been proposed as a regulator of Barx1 expression during tooth development (Tucker, Matthews et al. 1998). BMP4, a putative Hh target was shown to inhibit Barx1 expression. Moreover, exogenous inhibition of BMP4 by noggin resulted in ectopic Barx1 expression (Tucker, Matthews et al. 1998). Thus it remains to be determined if aberrant activation of Wnt signaling seen in human gastric cancers is a result of suppression of Barx1expression by Shh signaling.

Although the exact mechanisms by which cell patterning in the stomach is achieved remains vague, what is clear is that alterations of normal cellular proliferation and differentiation in the stomach contributes to preneoplastic changes in both mice and humans. Therefore understanding the regulatory

pathways active in the stomach during physiology and pathophysiology will help better understand disease etiology.

MATERIALS AND METHODS

Mice

Shh $^{LacZ/LacZ}$, mice have been described elsewhere(Jeong, Mao et al. 2004). These mice carry the β- galactosidase cDNA at the endogenous gene locus. The Shh $^{LacZ/LacZ}$ mice were maintained as a homozygous colony. The study was performed with the approval of the University of Michigan Animal Care and Use Committee, which maintains an American Association for Assessment and Accreditation of Laboratory Animal Care facility.

Bacterial Strain and Culture Conditions and Mouse infection

Helicobacter felis (ATCC, Manassas) was grown on Trypticase Soy Agar with 5% Sheep Blood, plates (BD Diagnostics BBL). Broth cultures were made by inoculating the bacteria in Brucella broth. Mice were gavaged orally inoculated three times over 3 days with 10^8 *H.felis* organisms in 100 μl of Brucella broth.

X-gal staining

The stomach was opened along the greater curvature and the gastric contents were washed in ice cold PBS. The stomach was fixed in fresh 4% paraformaldehyde/PBS (pH 7.0-7.5) for 1h at 4^{o}C, and then washed three times for 30 min each with β-gal rinse buffer (100 mM sodium phosphate pH 7.3, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40) at room temperature. The tissue was incubated for 16h at 37^{o}C in β-gal staining solution (β-gal rinse buffer,

25 mg/ml X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide). The tissue was rinsed with β-gal rinse buffer for 30 min at room temperature, and then post-fixed prior to paraffin-embedding and sectioning.

Immunofluorescence

Immunofluorescence staining was performed on 5 μm paraffin sections. Tissue sections were deparaffinized, rehydrated and blocked with 20% serum. A 1: 50 dilution of anti-Shh (Santa-Cruz) , a 1: 100 dilution of anti- CD45 (BD Pharmingen) , a 1: 100 dilution of anti-Muc6 (Novacastra Laboratories) and 1:800 dilution of mouse anti-H,K-ATPase (Medical & Biological Laboratories Co., LTD) antibodies were used on tissue sections, followed by a 1:500 dilution of fluorophore–labelled Alexafluor secondary antibody (Molecular probes). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, 1 μg/ml, Sigma) for tissue sections and images were generated on a Nikon Eclipse E800 microscope (Nikon, Melville, NY) using a SpotCD camera or confocal microscope.

Immunoblot analysis

Protein was loaded on a 4-20% SDS-PAGE gradient gel for immunoblot analysis. The membranes (Hybond-C Extra Nitrocellulose, Amersham Biosciences) were blocked with Detector Block (KPL, Gaithersburg, MD, 71-83-00) for 1h at room temperature followed by an overnight incubation with a 1:200 dilution of the goat polyclonal anti-Shh (Santa Cruz Biotechnology, sc-1194), or a 1h incubation with1:5000 GAPDH (Chemicon) antibody. The membranes were

washed twice for 10 min each in 1x TBST and incubated for 1h with a 1:5000 dilution of horseradish peroxidase-conjugated secondary anti-goat, or anti-mouse antibodies. The membranes were washed five times for 30 min with 1x TBST. Proteins were visualized using enhanced chemiluminescence (Lumilight substrate, Roche Applied Science, Mannheim, Germany).

Microarray analysis

RNA isolated from the antrums of the gastrin knockout and wild type mice were submitted for microarray analysis.

Immunohistochemical Staining

X-gal stained tissue sections were deparaffinized and rehydrated. The sections were then washed with 1x PBS, blocked with 20% serum and incubated with a 1: 1000 dilution of anti-activated β-catenin (BD Biosciences), for 1h. The primary antigen-antibody complex was detected with the avidin-biotin complexes using the Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine for the substrate (DAB, DAKO).

RESULTS

Shh expression retained in mucous cells after *Helicobacter* infection

In the normal glandular stomach Shh is expressed by both oxyntopeptic and mucous cell lineages. We have shown loss of Shh expression from parietal cells in both human subjects and mouse model of *Helicobacter* infection. During the progression from chronic inflammation to metaplasia the parietal cells are replaced by mucous cells. To determine the role of Shh in this process, we first established the expression pattern of Shh during the progression from inflammation to metaplasia in *Helicobacter* infected subjects. There were very few parietal cells per gland and these cells expressed low levels of Shh (Fig. 4.1A). However, the mucous cells occupied almost the entire gland and had retained Shh expression (Fig. 4.1A). We next investigated if the mouse model of *Helicobacter* infection recapitulates the human pattern of Shh expression. Tissue sections from Shh-LacZ mice infected for three weeks were co-stained for Shh expression (X-gal), and a parietal cell marker (H.K-ATPase), and a hematopoietic marker (CD45). The CD45 expressing hematopoietic cells were primarily seen in the submucosa but some glands showed invasion by these cells (Fig. 4.1B). In the glands where parietal cells were still present, there was a mixed population of both Shh-expressing and non-expressing parietal cells (Fig 4.1B, black arrow). Interestingly, comparable to the human pattern of Shh expression, we found intense Shh staining in the expanded mucous lineage cells (Fig. 4.1 B). Therefore, the loss of parietal cells resulted in an expansion of Shh-expressing mucous cells in the corpus.

Increase in Shh expression in the metaplastic antrum

Since mucous cells account for the major portion of the antral glands, we investigated the expression of Shh in the antrum of normal human, and *Helicobacter* infected human subjects with or without metaplasia. In the human gastric antrum, there was little Shh expression compared to the corpus. What expression was observed was primarily in the surface pit cells (Fig. 4.2 A). By contrast, in the *Helicobacter*-infected antrum without metaplasia, there was increased Shh expression in the gastric pit cells (Fig. 4.2 B). However in the human subjects with intestinal metaplasia there appeared patchy expression of Shh. Shh expression was absent in the gland with intestinal metaplasia, but the neighboring glands expressed Shh (Fig. 4.2 C). The gastrin knockout mice exhibit features described by Correa related to gastric cancer development, progressing from chronic inflammation to atrophy/metaplasia to antral tumors (Zavros, Eaton et al. 2005). Similar to the expression of Shh seen in human antrum, there was little expression of Shh in the normal mouse gastric antrum. However, the antral glands of the gastrin deficient mice showed a remarkable increase in the expression of Shh (Fig. 4.2 D). Thus, the antral glands under hypochlorhydric, atrophic conditions resulting from either *Helicobacter* infection or absence of gastrin, show an increase in expression of Shh.

Stromal sources of Shh expression in atrophic/metaplastic stomach

Fukaya et al. previously reported that Shh is expressed in the stroma, in intestinal type gastric cancer (Fukaya, Isohata et al. 2006). Therefore, we

investigated whether Shh is expressed in the stroma of *Helicobacter* infected human subjects and in the gastrin knockout mouse model. Our analysis of tissue sections from *Helicobacter* infected patients revealed expression of Shh in the lamina propria that colocalized with Muc6 expression (Fig. 4.3 A). TFF2 positive bone marrow derived cells have been reported in *Helicobacter* infected mice (Houghton, Stoicov et al. 2004). Therefore, we stained for the hematopoietic marker and found that these Shh expressing Muc6 positive cells in the stroma expressed the hematopoietic marker (CD45) (Fig. 4.3 B), suggesting that these cells could possibly contribute as one of the sources of Shh expression in the injured stomach. In the gastrin knockout mice, there was considerable Shh staining in the sub mucosal inflammatory infiltrate under the hyperplastic antral glands (Fig. 4.3 C). Elevated levels of IFNγ expression are reported in the antrum of gastrin knockout mice (Zavros, Eaton et al. 2005) therefore, we stained for IFNγ and found that the expression of Shh co-localized with IFNγ expression (Fig. 4.3 C). In fact, expression of Shh by resting and activated T-cells has been reported earlier (Outram, Varas et al. 2000; Bhardwaj, Murdoch et al. 2001; Lowrey, Stewart et al. 2002; Stewart, Lowrey et al. 2002).

Pro-inflammatory cytokines induce expression of Shh

Co-localization of Shh with IFNγ positive cells in gastrin null mice and an increase in Shh expression in the mucous cells from *Helicobacter*-infected subjects and mouse models raised the possibility that pro-inflammatory cytokines produced during *Helicobacter* infection or due to bacterial overgrowth might regulate Shh expression in mucous cells. To examine this possibility, we used

the NCIN87 cells which are a mucous carcinoma cell line. NCIN87 cells were cultured and treated with IFNγ, IL-1β, and TNFα for 6h and protein was prepared for western blot analysis. All three cytokines significantly induced Shh expression and processing (Fig. 4.4). However, there were differences in the protein among the three cytokines in their ability to induce Shh expression. IFNγ and IL-1β treatments increased the processed form, whereas TNFα seemed to increase the expression of the Shh precursor form (Fig. 4.4).

Reduced levels of Wnt antagonists in the antral tumor tissue from gastrin knockout mice

Generally, Shh signals from the epithelium to the mesenchyme. We referred to the microarray data comparing the expression of genes between wild type antrum, and antral tumors from gastrin knockout mice (Kang, Saqui-Salces et al. 2008), to look for mesenchymal genes that could influence epithelial organization.

During stomach development prior to gastric cell differentiation, there is extensive Wnt signaling in the endoderm. Mesenchymal derived Barx1 directs sFRP1 and sFRP2 expression thereby inhibiting Wnt and facilitating epithelial differentiation. Both sFRP1 and sFRP2 are known to antagonize Wnt signaling and have been shown to be sufficient to replace Barx1 activity (Kim, Buchner et al. 2005). We observed that there was a 2.6 fold decrease in the expression of Barx1 in the antral tumors of gastrin knockout mice (Fig. 4.5 A). Three different probe sets were used for sFRP1 in the microarray and we found that there was a

5.7, 3.4 and 3.3 fold decrease in the expression of sFRP1 in the antral tumors from gastrin knockout mice (Fig. 4.5 B), suggesting that the expansion in the mucous cell population could possibly be due to active Wnt signaling.

Active Wnt signaling in the metaplastic mucous cells

BMP4 is expressed in the interstitial mesenchymal cells of the adult stomach and is a putative Shh target gene (van den Brink, Hardwick et al. 2001). BMP4 inhibits expression of Barx1 has been shown to be important for normal tooth development. Inhibition of BMP4 by exogenous noggin leads to ectopic expression of Barx1, resulting in transformed tooth identity (Tucker, Matthews et al. 1998). Therefore, we hypothesized that an increase in Shh and its signaling results in increased levels of its putative target BMP4, which inhibits sFRP1 via inhibition of Barx1 leading to active Wnt signaling (Fig. 4.6). Indeed, we found accumulation of nuclear β-catenin in the mucous cells from *Helicobacter* infected subjects; mouse model and gastrin knockout mouse model (Fig 4.7 A-C). Interestingly, there was no nuclear β-catenin staining observed in any parietal cell.

DISCUSSION

In 1975, Correa proposed a human model of gastric neoplasia, in which chronic inflammation in the stomach leads to gastric atrophy, metaplasia and subsequently to dysplasia/cancer. Gastric atrophy is defined by the loss of parietal cells and chief cells. It is not clear if expansion in the mucous cell lineage is the cause or effect of gastric atrophy. In the milieu of chronic inflammation, aberrant increases in proliferation accompanied by impaired differentiation could explain the expansion in mucous cell populations. However, another possibility is that, the mucous cell expansion could just be an attempt to compensate for the loss of cells so as to maintain the cellular composition of stomach. Nevertheless, to prevent this “irreversible” step of atrophy leading to gastric cancer, understanding of the regulatory pathways active in the stomach during pathophysiology will help better understand disease etiology.

Accumulating evidence correlates Shh expression with gastric carcinogenesis (Berman, Karhadkar et al. 2003; Ma, Chen et al. 2005; Fukaya, Isohata et al. 2006; Wang, Choi et al. 2006; Lee, Han et al. 2007). Interestingly, both loss of Shh and its increase have been reported in the development of gastric cancer. Studies from several rodent models and human data showed loss of Shh both at mRNA and protein levels during *H. pylori* induced inflammation and atrophy. Interestingly, studies on human gastric cancer cell lines and human tissues indicate that the hedgehog pathway is frequently activated in advanced gastric adenocarcinoma and contributes to gastric cancer cell growth. Thus there is a gap in our understanding, how gastric atrophy

predisposes to cancer and the role played by the loss of Shh from the oxyntic cells and gain of Shh expression in the mucous cells.

Is Shh expression required for the survival of the cell producing it?

Prior to determining the functional role of Shh in the development of gastric cancer, its expression pattern needed to be determined. To address this, we studied sequential expression of Shh in different models of gastric cancer including *Helicobacter* infection model and the gastrin knockout mouse model. We found Shh expression was lost in the parietal cells prior to atrophy but its expression was retained in the mucous cells. This raised the question of whether expression of Shh is essential for a cell's survival. In other words, if we could maintain the expression of Shh in the parietal cells during gastric injury could we then prevent gastric atrophy. This could be addressed by over expressing Shh in the parietal cells and infecting the mice. However, one has to be careful using the H, K-ATPase promoter as a driver for Shh, since chronic inflammation down regulates its expression. Hence for these purposes a conditional epithelial-cell specific promoter might be used. Although, the use of H, K-ATPase promoter to drive Shh expression in mice with *Helicobacter* infection apart from helping identify the role of Shh in the stomach, can address an important unanswered question in the field. Does Shh over expression overcome the loss of H, K-ATPase expression seen with infection as suggested by the canine parietal cell data (Stepan, Ramamoorthy et al. 2005).

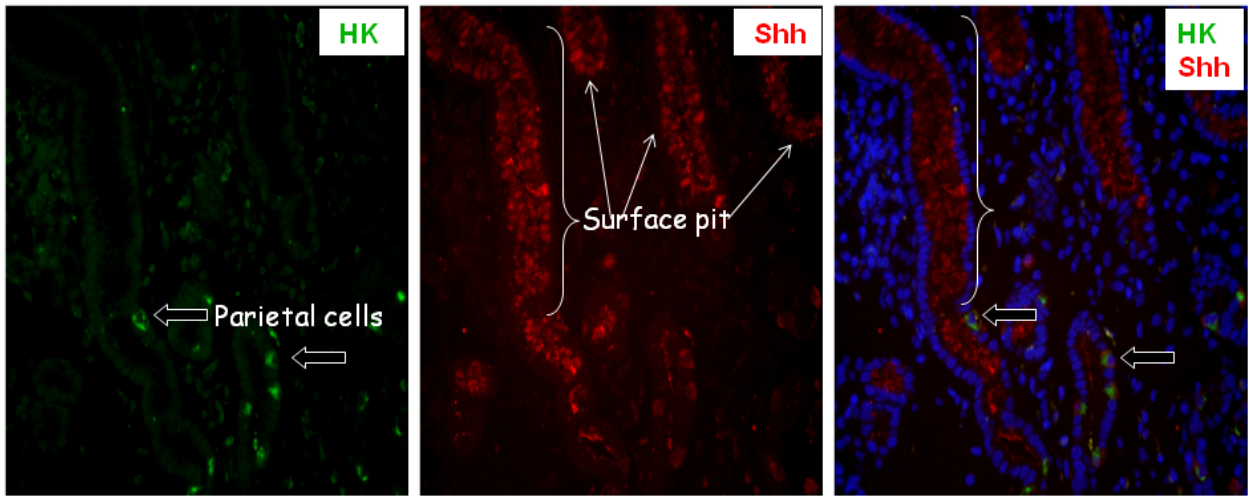
Is parietal cell Shh different from the mucous cell Shh?

Since Shh is a secreted protein, and signals in a paracrine manner, then why would loss of Shh specifically from parietal cells lead to parietal cell atrophy and mucous cell expansion? While the parietal cells lose Shh expression there is still a pool of Shh being expressed in the stomach. There are several hypotheses that could be generated. Firstly “in an infected hypochlorhydric stomach Shh is not processed”. We have shown earlier that pepsin is important for processing of Shh to the 19kDa active form (Zavros, Waghray et al. 2007). Therefore, it remains to be determined if the inflamed stomach produces only precursor (45kDa) form and if that had any biological activity. Nonetheless we have preliminary data suggesting a role of inflammatory cytokines in inducing the processing of Shh. Secondly, “is the direction of Shh secretion different between parietal and mucous cells”. In the recent studies it has been shown that Shh is secreted apically from the parietal cells (Zavros, Orr et al. 2008). Therefore, during atrophy even though there might be increased Shh expression in the mucous cells it might not be available to be received by the responding cell. Based on the Gli1 expression the cells responding to Hh are the mesenchymal cells. It still remains to be determined how Hh produced in the apical surface reaches the basolateral side of the cell. Thirdly, “differentiated parietal cells lack the receptors for proliferating signals”. Shh from the epithelial cells might induce the expression of proliferation signals in the mesenchyme including the production of Wnts. However, it is not known if parietal cells are capable of responding to Wnt signals.

Active Wnt signaling in human gastric cancer

Wnt signaling is suggested to play a role in gastric carcinogenesis. Fifty four percent of human gastric cancer tissues show active Wnt signaling (Oshima, Matsunaga et al. 2006). Over- expression of Wnt1 in the gastric mucosa resulted in suppression of epithelial differentiation and development of preneoplastic lesions consisting of undifferentiated cells (Oshima, Matsunaga et al. 2006). Barx1 is a transcription factor, strongly expressed in the mesenchymal wall of the developing stomach. Barx1 null mice have a disorganized gastric epithelium and show intestinal gene expression in the stomach. Barx1 is known to induce the expression of sFRP1 and sFRP2 factors that antagonize Wnt signaling. This inhibition of Wnt signaling was shown to be critical for normal stomach development (Kim, Buchner et al. 2005). We speculate that increased levels of Shh signaling inhibit the expression of Barx1 thereby facilitating active Wnt signaling. To this end we have detected active nuclear β-catenin expression in the mucous cells in models with high Shh expression. Interestingly, we did not detect any nuclear β-catenin expression in the parietal cells. It remains to be determined if the putative Hh target BMP4 inhibits Barx1 in stomach, and which Wnts participate in gastric carcinogenesis.

A



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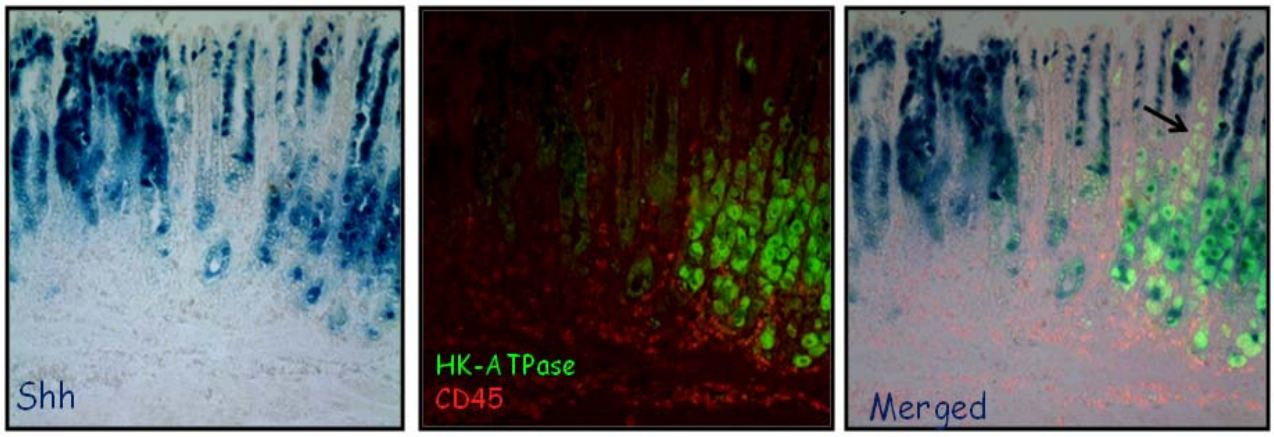


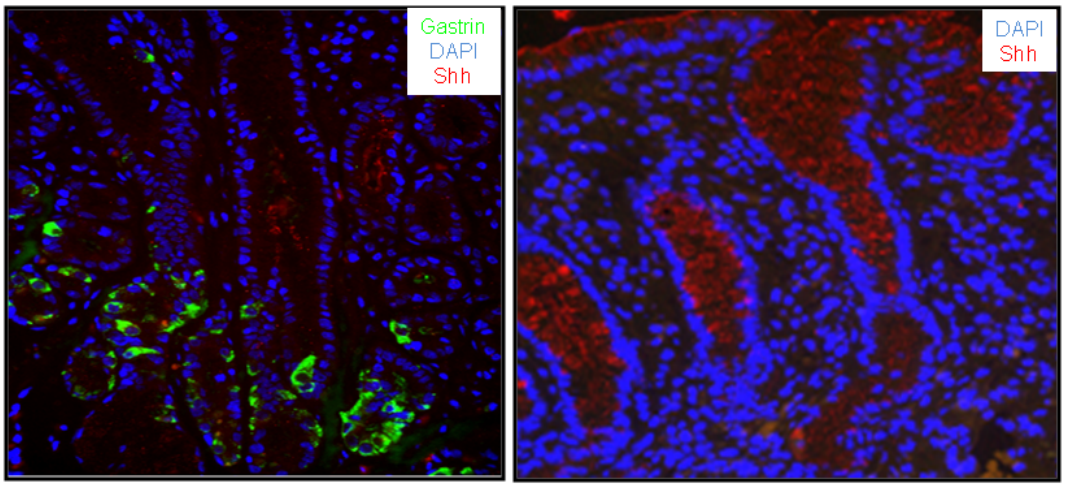
Fig. A.1: *Helicobacter* infection induces Shh expression in mucous cells in gastric corpus

A. Human stomach sections from subjects infected with *H. pylori* were examined by immunofluorescence for Shh expression. In the atrophic gastric corpus Shh expression was increased in the mucous cells. The β-subunit of H, K,-ATPase was used to detect the parietal cells (FITC, green), Shh (red) and the nuclei are stained with DAPI (blue). The merged view is shown. B. Immunohistochemical staining of parietal cells and hematopoietic cells on X-gal stained paraffin sections from mice infected for three weeks *H.felis*. Intense Shh staining was seen in the mucous cells. The β-subunit of H, K,-ATPase was used to detect the parietal cells (FITC, green); CD45 (red) and the nuclei are stained with X-gal (blue). The merged view is shown. Arrow indicates absence of Shh (X-gal) expression in parietal cells.

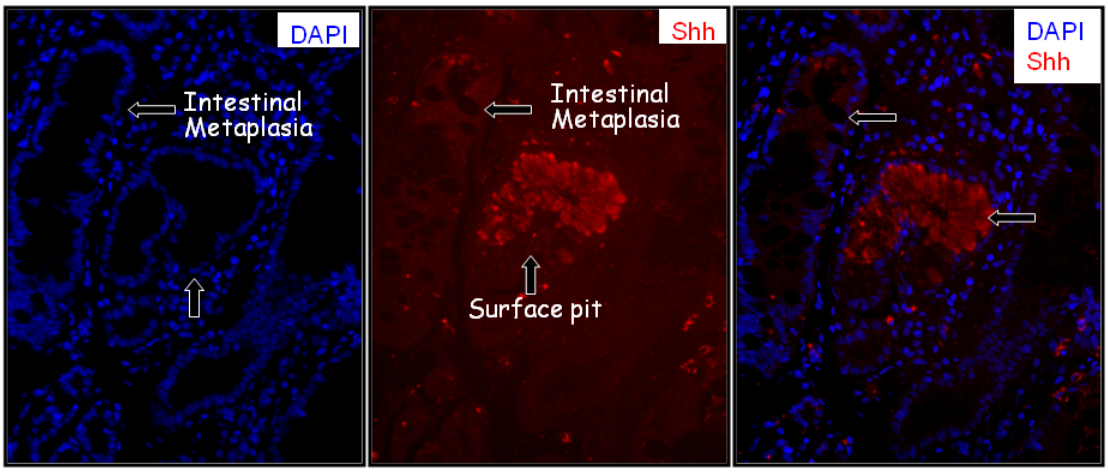
Fig. A.2: Increased Shh expression in gastric antral glands of hyperplastic/metaplastic stomach.

A. Shh expression in the normal human antrum was detected by immunofluorescence. The antrum stained for gastrin (FITC-green), Shh (Texas red) and nuclei (DAPI-blue). Human antral section from *Helicobacter* infected patient without metaplasia was examined for Shh expression. The surface pit glands show an increase in Shh expression (Texas red), the nuclei are stained with DAPI (blue). B. Expression of Shh was examined in human antral metaplastic tissue sections. Shh expression was seen in the "non-metaplastic" neighboring glands (Texas red). No Shh expression was detected in swiss cheese appearing intestinal metaplastic glands. Merged image is shown; nuclei were stained with DAPI-blue. C. Stomach sections from normal and gastrin knockout mice were probed for Shh. Wild type antrum shows a faint Shh staining in mucous cells. The right panel shows increased Shh staining in the mucous glands of the gastrin knockout antrum. The insert is a high power version of the surface pit region in the antrum. Shh was localized using Texas red-labeled secondary (red). Nuclei are stained with DAPI.

A


Gastrin
DAPI
Shh
DAPI
Shh

B


DAPI
Intestinal Metaplasia
Shh
Intestinal Metaplasia
Surface pit
DAPI
Shh

C

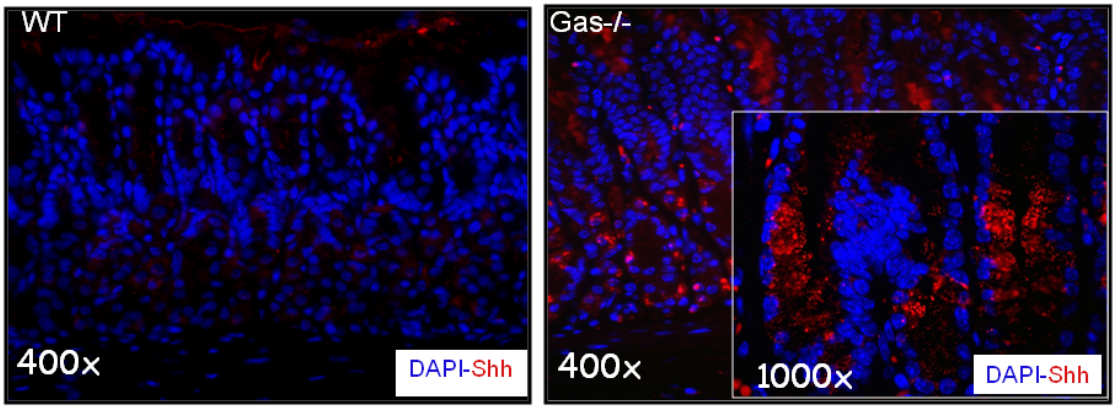
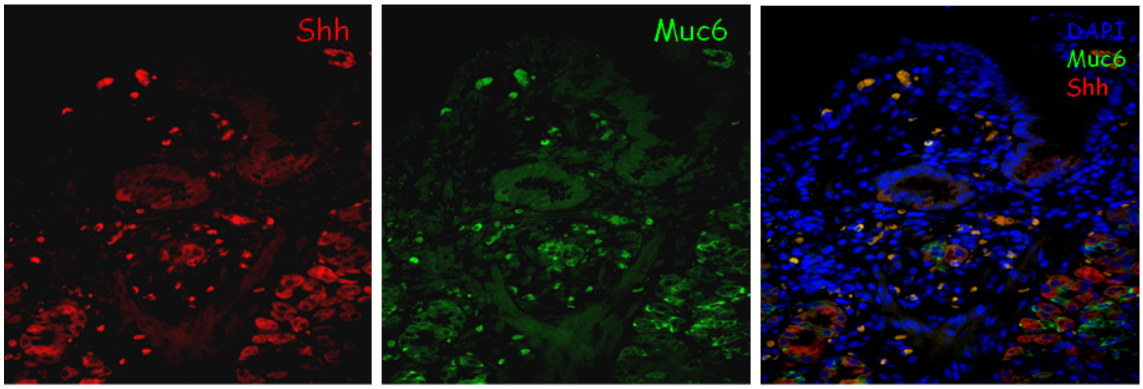

WT
400x
DAPI-Shh
Gas-/-
400x
1000x
DAPI-Shh

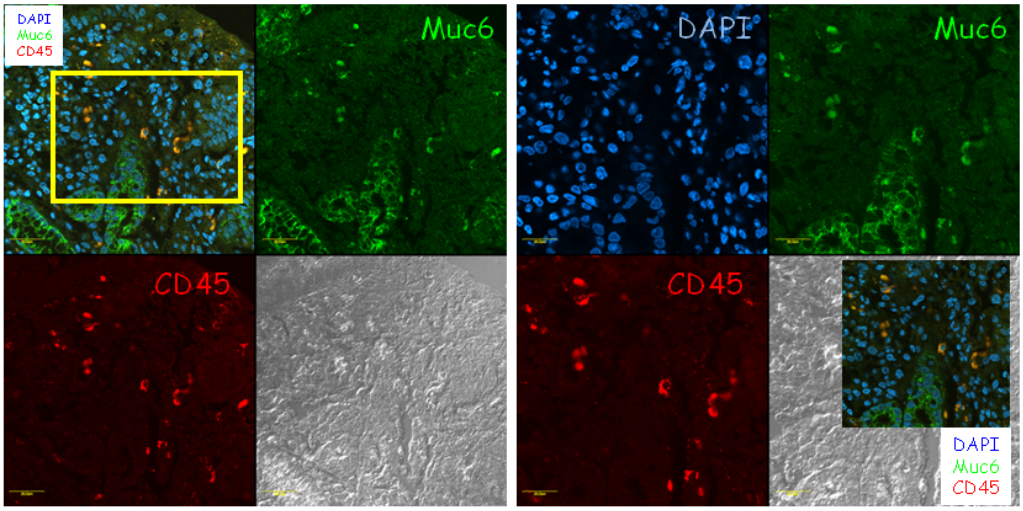
Fig. A.3: Stromal expression of Shh in proximity to inflammation

A. Confocal staining was performed on tissue sections from *Helicobacter* infected human subjects for Shh expression. Parietal cells and cells in the lamina propria stained for Shh (Texas red). Shh positive cells in the stroma were also positive for the mucous neck cell marker Muc6 (FITC-green). Muc6 expression was also seen in the corpus glands. Nuclei were stained with DAPI (blue), merged image is shown. B. Confocal staining showing expression of CD45 and Muc6. Muc6 positive cells (FITC-green) in the stroma were also positive for the hematopoietic marker CD45 (red). Merged image is shown. The image on the right is high power view of the boxed area. C. Shh expression in the inflammatory cells. H&E of the gastrin knockout mice hyperplastic glands is shown. The submucosal inflammation is indicated (rectangle). Shh (Texas red) and IFNγ (FITC-green) expression is shown within the inflammation. Merged panel shows an overlap of Shh and IFNγ expressing cells within the infiltrate (yellow).

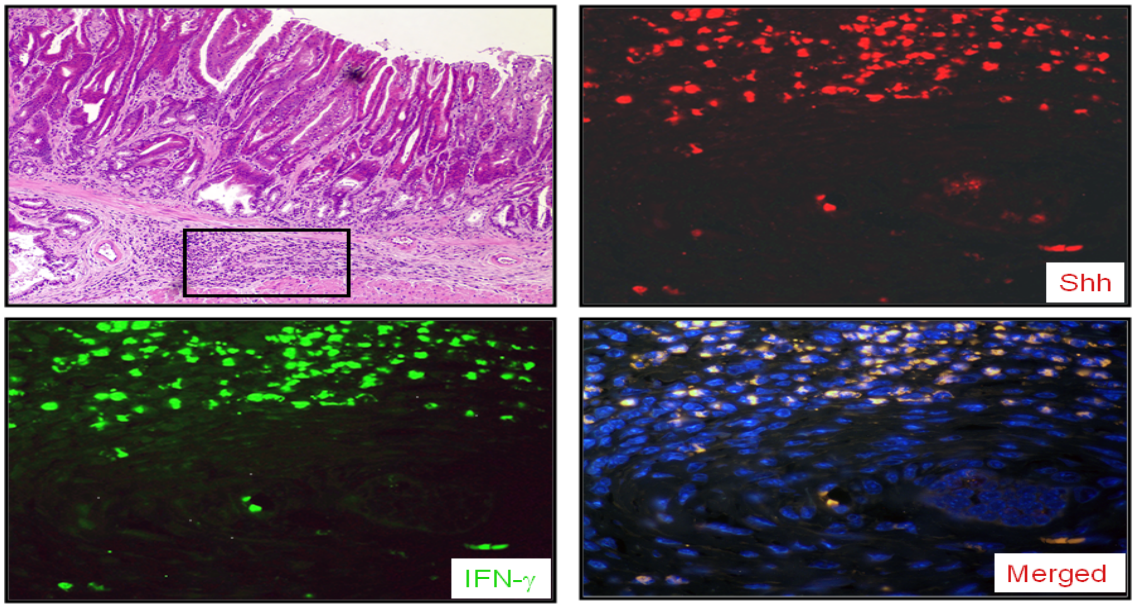
A


Shh
Muc6
DAPI
Muc6
Shh

B


DAPI
Muc6
CD45
Muc6
DAPI
Muc6
CD45
CD45
DAPI
Muc6
CD45

C


Shh
IFN-γ
Merged

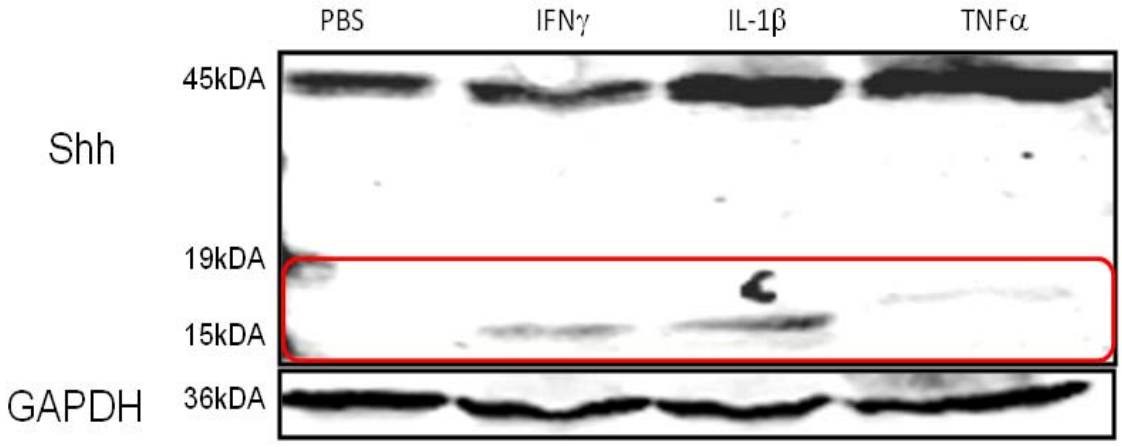


Fig. A.4: Pro-inflammatory cytokines induce Shh expression in mucous cell line

NCI-N87 cells were stimulated with IFNγ (100ng/ml), IL-1β (100ng/ml) and TNFα (100ng/ml) or PBS for 6h. Cell lysates were collected in RIPA buffer and resolved on a gradient gel for western blot analysis. Both the precursor form and the processed form (rectangle) were detected. The blot was reprobed for GAPDH.

A

Normalized expression level	WT Antrum	G-/- Tumor Antrum	Fold Change
Barx1	7.955428	6.541810	-2.66

B

Normalized expression level	WT Antrum	G-/- Tumor Antrum	Fold Change
sFRP1	7.398707	4.870256	-5.77
sFRP1	7.162950	5.372254	-3.46
sFRP1	8.272912	6.525611	-3.36

Fig. A.5: Microarray data comparing gene expression in wild type and gastrin knockout mice from (Kang, Saqui-Salces et al. 2008)

A. Shows normalized expression and fold change in Barx1 between wild type antrum and antral tumor from gastrin knockout mice. B. Shows normalized expression of sFRP1 from three different probes comparing expression between normal antrum and antral tumor.

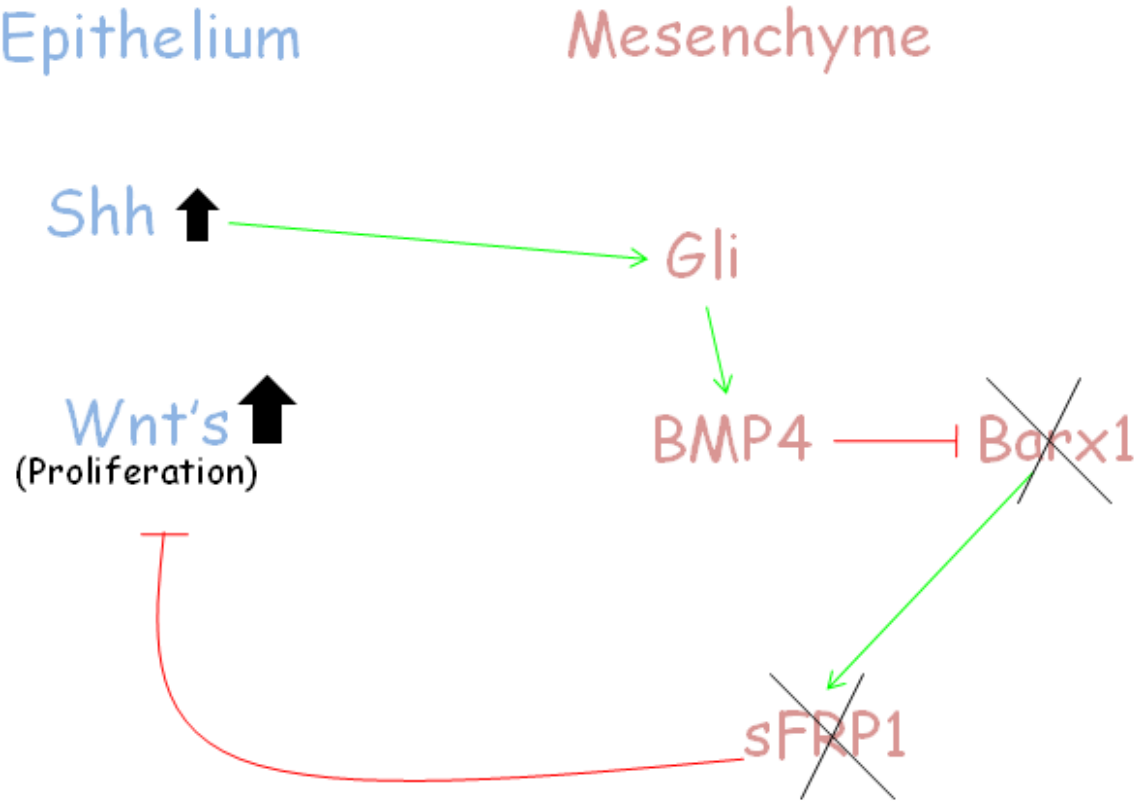


Fig. A.6: Working model showing the relationship between epithelial Shh and mesenchymal Wnt

Epithelial Shh induces the expression of its putative target BMP4 in the mesenchyme. BMP4 inhibits the mesenchymal expression of Barx1 which is required to suppress regional Wnt activity via sFRP1. In the presence of high levels of Shh, there are enhanced BMP4 levels resulting in reduced expression of Barx1 and therefore sFRP1, leading to high Wnt signaling, and proliferation.

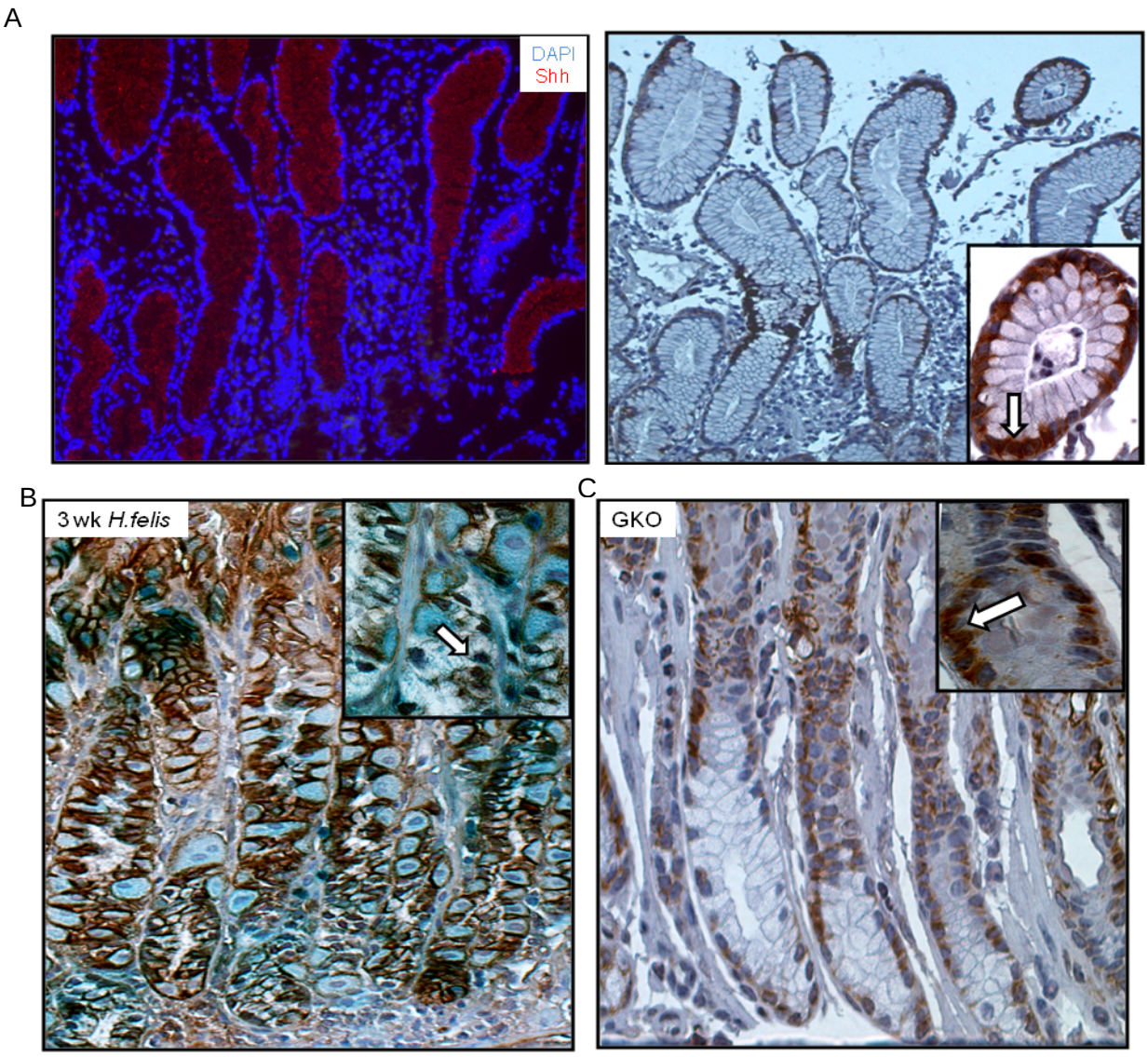


Fig. A.7: Activated Wnt signaling in different models of mucous cell metaplasia

A. Metaplasitc human tissue sections were stained for Shh. Expression of Shh was detected in the metaplastic mucous glands (Texas red), nuclei were stained with DAPI (blue) merged image is shown. The same section was re-probed with activated β-catenin antibody. Inset is the high power view, arrow shows nuclear staining of b-catenin. B. Sections from 3 wks H.felis infected Shh-LacZ mice were stained for β-catenin. X-gal (blue) shows Shh expression and nuclear β-catenin is brown. Inset is a high power view and the arrow indicates nuclear localization of β-catenin in mucous cells. C. Nuclear β-catenin staining of hyperplastic antrum from a gastrin knockout mice. Insert in a high power view of the antral gland and arrow indicates nuclear staining of β-catenin.

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